

ASPECTS OF PHOSPHORUS NUTRITION  
IN ENDOMYCORRHIZAL FUNGI OF THE  
ERICACEAE

BY

COLIN JOHN STRAKER

A thesis presented for the degree of Doctor of Philosophy  
in the Faculty of Science,  
University of Cape Town

Department of Botany

January 1986

The University of Cape Town has been given  
the right to reproduce this thesis in whole  
or in part. Copyright is held by the author.

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

ABSTRACT

An investigation was undertaken on the phosphorus nutrition of the ericoid endophytes isolated from the root systems of Vaccinium macrocarpon, Aiton, Rhododendron ponticum L., Calluna vulgaris (L.) Hull, Erica hispidula L., and E. mauritanica L. These endophytes were grown on a liquid medium containing inositol hexakisphosphate, fractionated into cytoplasmic, extracellular and wall- and membrane-bound fractions and acid phosphatase activity was assayed using p-nitrophenyl phosphate as the substrate. The presence of extracellular phosphatase was demonstrated in all cultures seven days after inoculation, but its activity was highest in the endophyte of E. hispidula. The wall- and membrane-bound acid phosphatase was the dominant fraction in the European endophytes. The endophyte of E. hispidula, growing in a medium containing high and low levels of sodium inositol hexakisphosphate was fractionated into cytoplasmic, extracellular, membrane-bound and wall-bound fractions with the wall-bound enzymes being solubilised by 1 M NaCl. In high P-fed mycelia the extracellular acid phosphatase had the greatest activity (75% of total activity) 12 days after inoculation whereas in low P-fed mycelia both soluble-wall and extracellular fractions contributed equally to form 80% of total activity. When the fractions were eluted through a Sephacryl S-400 gel filtration column, similar single acid phosphatase peaks were obtained from all the fractions

except the soluble-wall fraction from low P-fed mycelia which produced an additional peak representing a lower molecular weight phosphatase. The molecular weights corresponding to the main peak common to all fractions and the lower molecular weight peak were 173 858 and 68 028 respectively. The pH optimum of the low molecular weight phosphatase was pH 6.5 whereas the high molecular weight phosphatases showed a broad optimal range between pH 2.0 and 6.0. The enzymes did not show a specific requirement for metal ions and there were variations in response to different compounds. All the enzymes were inhibited by fluoride, molybdenum, arsenate, cyanide, mercury and phosphate but stimulated by EDTA, citrate and the ferric ion in low concentrations. The phosphatases showed a wide substrate specificity with their maximum affinity for inorganic pyrophosphate and high affinities for  $\alpha$ - and  $\beta$ -naphthyl phosphate, phenyl phosphate and  $\alpha$ -glycerophosphate but a low affinity for phytic acid. The low molecular weight enzyme was the only one with the ability to hydrolyse the organic anhydrides, ATP, ADP and AMP.

Mycelia of the endophyte of E. hispidula were grown in liquid culture media at high and low levels of orthophosphate and phosphate uptake rates were measured over a wide concentration range. The kinetic constants, (i.e.  $V_{max}$  and  $K_m$ ) were estimated by the Direct Linear Plot method using a computer based analysis and a dual uptake

system was demonstrated. In the low-affinity system,  $V_{max}$  values of low P-fed and high P-fed mycelia were similar whereas the  $K_m$  of high P-fed mycelia was lower than that of low P-fed mycelia. In the high-affinity system, the  $K_m$  values of high P-fed and low P-fed mycelia were similar whereas high P-fed mycelia showed a higher  $V_{max}$  value than low P-fed mycelia. In low P-fed mycelia, the low-affinity contribution to total uptake was 93% at 500  $\mu$ M and 25% at 1  $\mu$ M external phosphate. The uptake systems were sensitive to the pH of the incubation medium and were inhibited by 2,4-dinitrophenol. The endophyte showed linearity of absorption for only 1 min and absorption rates were higher in mycelia grown on very low levels of orthophosphate.

Metachromatic staining demonstrated the presence of polyphosphate granules in endophytes isolated from root systems of V. macrocarpon, R. ponticum, C. vulgaris, E. hispidula and E. mauritanica. The granules accumulated in response to high concentrations of phosphorus in the external medium and during the lag phase of growth. Nucleic acid-polyphosphate co-precipitates prepared from endophytes were separated by means of polyacrylamide gel electrophoresis and the molecular weights of polyphosphate of the endophytes of E. hispidula, E. mauritanica and R. ponticum were between 3000 and 4700. Inoculated root systems of V. macrocarpon had significantly more acid-labile polyphosphate than non-mycorrhizal roots.

Mycelia of the endophyte of E. hispidula, grown on high levels of organic P accumulated high amounts of acid-soluble and acid-insoluble polyphosphate precipitated by  $\text{BaCl}_2$ . Under conditions of P deprivation the polyphosphate fractions declined whereas the orthophosphate fractions increased. Negligible amounts of polyphosphates accumulated in low P-fed mycelia.  $^{32}\text{P}$  fractionation studies indicated the acid-insoluble polyphosphate fraction to be higher than the acid-soluble one.

The results of these investigations are discussed in relation to the phosphorus nutrition of ectomycorrhizas and VA mycorrhizas and the importance of ericoid mycorrhizas in the phosphorus nutrition of heathlands.

ACKNOWLEDGEMENTS

I would like to express my appreciation to the following people who in various ways have contributed to the completion of this thesis.

My supervisor, Professor Derek Mitchell for initially suggesting the project and for his guidance, interest and support and thorough scrutiny of the manuscript.

Professor O.A.M. Lewis for permission to use the facilities of the Botany department.

Professor Eugene Moll for allowing me desk space in the Eco-lab.

Mr Eric O'Neill of the Department of Microbiology for much appreciated assistance with the gel electrophoresis of the acid phosphatases and Professor D. Woods for permission to use the department's equipment.

Dr Mike Picker of the Zoology department for initial advice on the gel electrophoresis of polyphosphates.

Nicholette Allsopp and Dr Karl Schütte for useful discussions and advice at various stages of the project.

Dr Donald Burns of the Department of Scientific and Industrial Research, Auckland, New Zealand, for allowing me to use his Double-Hyperbola Curve Fit computer programme.

Hilton Nye for so efficiently modifying the Double-Hyperbola Curve Fit programme.

Dr Tim Dunne for performing the Correspondence Analysis of polyphosphate granule size classes.

Dr D.J. Read of Sheffield University for allowing me the use of the European isolates.

My post-graduate colleagues, Pat Beeston, Sue Brown, Tony Cunningham, Craig Hilton-Taylor, Clive McDowell, Natasha Romoff and Willy Stock for their 'camaraderie' and friendship.

Tony Walsh for proof-reading some chapters of the manuscript and assisting with the arduous task of the reference list and my parents for their support and financial assistance during lean times.

I gratefully acknowledge financial support from the University of Cape Town and CSIR, South Africa.



<u>CONTENTS</u>	<u>PAGE</u>
ABSTRACT	(i)
ACKNOWLEDGEMENTS	(v)
CONTENTS	(vii)
PREFACE	(xiv)
LIST OF TABLES	(xvi)
LIST OF FIGURES	(xviii)
 CHAPTER 1 : INTRODUCTION	 1
1.1 NITROGEN NUTRITION	6
1.2 PHOSPHORUS NUTRITION	7
1.2.1 Access to unavailable sources of Phosphorus	9
1.2.2 Phosphate absorption	12
1.2.3 Phosphate storage	17
 CHAPTER 2 : MATERIALS AND METHODS	 24
2.1 CULTURAL PROCEDURES	24
2.1.1 Isolation of endophytes	24
2.1.2 Synthesis of mycorrhizal root systems	24
2.1.3 Preparation of liquid cultures	26
2.1.4 Growth of the endophytes of <u>Erica hispidula</u> and <u>E. mauri-</u> <u>tanica</u> in culture	27

	<u>PAGE</u>
2.2 THE EXTRACTION, FRACTIONATION AND CHARACTERIZATION OF THE ACID PHOSPHATASES OF ISOLATED ENDOPHYTES	28
2.2.1 Fractionation of mycelia of endophytes to yield extra- cellular, wall/membrane-bound and cytoplasmic factions	28
2.2.2 Fractionation of mycelia of the endophyte of <u>Erica hispidula</u> prior to partial purification and characterization of acid phos- phatases	29
2.2.3 Gel filtration	30
2.2.4 Acid phosphatase assay	31
2.2.5 Protein determinations	33
2.2.6 Phosphorus assay	33
2.2.7 Gel electrophoresis	35
2.3 THE KINETICS OF PHOSPHATE UPTAKE BY THE ENDOPHYTE OF <u>ERICA HISPIDULA</u>	37
2.3.1 General incubation and radio- isotope counting procedures	37
2.3.2 Efflux studies	40
2.3.3 Derivation of kinetic parameters	40
2.4 IDENTIFICATION, EXTRACTION AND ESTIMAT- ION OF POLYPHOSPHATES AND PHYTIC ACID	44

	<u>PAGE</u>
2.4.1 Cytochemical methods for the identification of polyphosphates	44
2.4.2 Phenol-detergent extraction of undegraded nucleic acid-polyP co-precipitates	44
2.4.3 Polyacrylamide gel electro- phoresis	46
2.4.4 Total P and acid-labile polyP determinations	46
2.4.5 Statistical analysis	47
2.4.6 Phosphorus extraction and fractionation procedures	48
2.4.7 $^{32}\text{P}$ absorption and utilisation by mycelia in culture	48
2.4.8 Total P determination of non- radioactive fractions	48
2.4.9 The effect of activated charcoal on the adsorption of P contaminants of polyP fractions	50
2.4.10 Phytic acid determination	50
 CHAPTER 3 : ACID PHOSPHATASE ACIVITY IN ISOLATED ENDOPHYTES	 51
 3.1 INTRODUCTION	 51
 3.2 RESULTS	 52

	<u>PAGE</u>
3.2.1 Growth curves of the South African endophytes	52
3.2.2 Acid phosphatase activity and protein content of mycelial fractions of European and a South African endophyte	52
3.2.3 Phosphatase activity in fractions of mycelia of the endophyte of <u>E. hispidula</u>	57
3.2.4 Gel filtration	60
3.2.5 pH scans	63
3.2.6 The action of effectors	63
3.2.7 Substrate specificity	66
3.2.8 Gel electrophoresis	69
3.3 DISCUSSION	69
CHAPTER 4 : KINETICS OF PHOSPHATE UPTAKE BY THE ISOLATED MYCORRHIZAL ENDOPHYTE OF <u>E. HISPIDULA</u>	76
4.1 INTRODUCTION	76
4.2 RESULTS	77
4.2.1 Uptake of $^{32}\text{P}$ by mycelia grown on different concentrations of P	77

	<u>PAGE</u>
4.2.2 The rate of uptake of $^{32}\text{P}$ by mycelia	77
4.2.3 Efflux studies	81
4.2.4 Preliminary experiments on the rate of uptake by mycelia in relation to P concentration	83
4.2.5 The effect of pH on P uptake kinetics	83
4.2.6 The effect of a metabolic inhibitor on uptake	88
4.2.7 The kinetic parameters of dual- system uptake in high P- and low P-fed mycelia	88
4.3 DISCUSSION	94
CHAPTER 5 : THE IDENTIFICATION, EXTRACTION AND FRACTIONATION OF POLYPHOSPHATES AND PHYTIC ACID	101
5.1 INTRODUCTION	101
5.2 RESULTS	102
5.2.1 Cytochemical observations of polyP granules	102
5.2.2 Polyacrylamide gel electro- phoresis for separation of	106

	<u>PAGE</u>
nucleic acid-polyP	
co-precipitates	
5.2.3 P and acid-labile polyP content	110
in phenol-detergent extracts	
5.2.4 The effect of P starvation on	112
the endogenous P status of	
isolated endophytes of <u>E.</u>	
<u>hispidula</u> grown on high and low	
levels of orthophosphate	
5.2.5 Activated charcoal adsorption of	114
possible contaminants of BaCl <sub>2</sub>	
precipitates	
5.2.6 Phytic acid content of mycelia	114
of the endophyte of <u>E. hispidula</u>	
5.2.7 Fractionation of <sup>32</sup> P in mycelia	116
of the endophyte of <u>E. hispidula</u>	
after incubation in KH <sub>2</sub> <sup>32</sup> PO <sub>4</sub> and	
various concentrations of P	
5.2.8 Fractionation of <sup>32</sup> P over time	119
in mycelia of the endophyte of	
<u>E. hispidula</u> grown on high and	
low levels of orthophosphate	
5.3 DISCUSSION	121
CHAPTER 6 : GENERAL DISCUSSION	127

	<u>PAGE</u>
REFERENCES	133
APPENDICES	145
APPENDIX I    DOUBLE-HYPERBOLA CURVE FIT COMPUTER PROGRAMME	145
APPENDIX II  REPRINT OF PAPER ENTITLED 'THE CHARACTERIZATION AND ESTIMATION OF POLYPHOSPHATES IN ENDOMYCORRHIZAS OF THE ERICACEAE'. <u>THE NEW PHYTOLOGIST</u> (1985) 99, 431-440	147

PREFACE

Soil nutrient levels and the nutrient cycling processes of the heathlands of the south-western Cape, South Africa have recently been the subject of an intensive investigation under the auspices of the Fynbos Biome Programme, CSIR. A major interest has been the phosphorus status of fynbos soils, phosphorus distribution patterns within plants, litter production and decomposition studies and the response of the vegetation to the addition of nutrients. Little is known of the phosphorus nutrition of ericaceous plants or their associated mycorrhizas in the fynbos biome. This project is a study which investigates aspects of the phosphorus nutrition of endophytes isolated from root systems of indigenous ericas. On the basis of other mycorrhizal studies, three main aspects were selected for investigation:

- 1) to investigate the acid phosphatase activity of isolated endophytes with a view to assessing the role of these enzymes in the utilization of organic P substrates in the soil
- 2) to investigate the phosphorus uptake processes of the endophyte and establish its efficiency in absorbing free phosphate ions from the external medium



3) to establish the potential of the mycorrhizas to store phosphorus in the form of polyphosphates, especially in times of excess P availability.

In view of the differences both in climate and soil nutrient levels between South African and European heathlands and the availability of isolated European endophytes from Dr. D.J. Read of Sheffield University, some comparative studies were undertaken between South African and European endophytes.

This thesis is structured into six chapters. The Introduction comprises a review of the literature and states the aims and objectives of the study. This is followed by the Materials and Methods and then three chapters dealing with one aspect of the study, each forming the basis of one paper. Part of Chapter 5 has already been published as "The Characterization and Estimation of Polyphosphates in Endomycorrhizas of the Ericaceae" by C.J. Straker and D.T. Mitchell in The New Phytologist (1985) 99, 431-440. The thesis concludes with a General Discussion in Chapter 6.

<u>LIST OF TABLES</u>	<u>PAGE</u>
1.1 Kinetic constants of the phosphate uptake systems of excised mycorrhizas, fungi and excised plant roots.	16
3.1 Acid phosphatase activity in fractions of mycelia of the endophyte of <u>Erica hispidula</u> .	59
3.2 The influence of various reagents on the acid phosphatase activity of fractions of mycelia of the endophyte of <u>Erica hispidula</u> .	65
3.3 The affinity of acid phosphatase enzymes of fractions of mycelia of the endophyte of <u>Erica hispidula</u> towards various substrates.	67
4.1 The effect of incubation in solutions of increasing concentration of orthophosphate on the flux of $^{32}\text{P}$ absorbed by mycelia of the endophyte of <u>Erica hispidula</u> .	82
4.2 The influence of pH on the dual-system kinetic parameter estimates of the P uptake systems of mycelia of the endophyte of <u>Erica hispidula</u> .	87

PAGE

- 4.3 Dual-system kinetic parameter estimates of the P uptake systems of high P-fed and low P-fed mycelia of the endophyte of Erica hispidula. 93
- 5.1 Acid-labile polyP content of phenol-detergent extracts and total P of isolated endophytes and aseptically infected root systems of Vaccinium macrocarpon. 111
- 5.2 The effect of the addition of activated charcoal to TCA-soluble and -insoluble extracts on P fraction levels of mycelia of the endophyte of Erica hispidula. 115

<u>LIST OF FIGURES</u>	<u>PAGE</u>
1.1 Diagram of the polyphosphate cycle	21
2.1 Cortical cells of seedlings of <u>Vaccinium macrocarpon</u> infected with the endophytes of <u>Erica mauritanica</u> and <u>Erica hispidula</u>	25
2.2 Standard curve showing the relationship between absorbance at 410 nm and $\mu\text{mol}$ p-nitrophenol	32
2.3 Standard curve showing the relationship between absorbance at 500 nm and 750 nm and $\mu\text{g}$ protein (Lowry <u>et al.</u> , 1951)	34
2.4 Standard curve showing the relationship between absorbance at 882 nm and $\mu\text{mol}$ phosphorus (Murphy & Riley, 1962)	36
2.5 Curves used for the correction of radioactive counts (cpm) due to quenching	39
2.6 Standard curve showing the relationship between absorbance at 689 nm and $\mu\text{mol}$ phosphorus (Kempers, 1975).	41

2.7 Generalised plot of the Hofstee linear transformation of the Michaelis-Menten equation	43
2.8 Summary of the phenol-detergent extraction procedure for nucleic acid-polyP co-precipitates	45
2.9 Summary of the phosphorus extraction and fractionation procedure based on that of Aitchison & Butt (1973)	49
3.1 Growth curves of cultures of the endophyte of <u>Erica hispidula</u> and <u>Erica mauritanica</u> on inorganic and organic P	53
3.2 Protein levels in cytoplasmic, extracellular and wall/membrane-bound fractions of four endophytes	55
3.3 Acid phosphatase activity of cytoplasmic, extracellular and wall/membrane fractions of four endophytes	56
3.4 Effect of NaCl on the release of acid phosphatase from the cell wall of the endophyte of <u>Erica hispidula</u>	58

3.5 Elution profiles of protein and acid phosphatase following gel filtration (Sephacryl S-400) of fractions of the endophyte of <u>Erica hispidula</u>	61
3.6 Molecular weight estimation by gel filtration of the acid phosphatase enzymes of the endophyte of <u>Erica hispidula</u>	62
3.7 pH scans of phosphatase activity in fractions of the endophyte of <u>Erica hispidula</u>	64
3.8 Electrophoretic mobility of the acid phosphatases of fractions of the endophyte of <u>Erica hispidula</u>	68
4.1 Uptake of $^{32}\text{P}$ by mycelia of the endophyte of <u>Erica hispidula</u> grown on a basal medium containing various concentrations of orthophosphate	78
4.2 Phosphate uptake by mycelia of the endophyte of <u>Erica hispidula</u> with time	79
4.3 The rate of uptake of $\text{KH}_2^{32}\text{PO}_4$ and glucose-6-phosphate ( $^{32}\text{P}$ ) over time by mycelia of the endophyte of <u>Erica hispidula</u>	80

PAGE

- 4.4 Double reciprocal plots of rate of uptake vs concentration by mycelia of the endophyte of Erica hispidula 84
- 4.5 Hofstee plots showing the effect of pH on high-affinity and low-affinity P uptake systems in mycelia of the endophyte of Erica hispidula 86
- 4.6 Hofstee plot of rate of uptake vs P concentration for mycelia of the endophyte of Erica hispidula grown on 6 mM orthophosphate 90
- 4.7 Hofstee plot of rate of uptake vs P concentration for mycelia of the endophyte of Erica hispidula grown on 0.06 mM orthophosphate 91
- 4.8 Diagram showing the relative contributions of the high- and low-affinity uptake systems in 6 mM- and 0.06 mM P-fed mycelia of the endophyte of Erica hispidula 92
- 5.1 A hypha of the mycelium of the endophyte of Rhododendron ponticum stained with toluidine blue (pH 1.0) to show vacuolar granules of polyphosphate 103

5.2 The percentage of metachromatic granules in relation to different size classes in 10-d-old mycelia of isolated endophytes	104
5.3 Numbers of metachromatic granules in the endophyte of <u>Vaccinium macrocarpon</u> grown on various sources of P	105
5.4 Frequency of metachromatic granules during growth of the endophyte of <u>Vaccinium macrocarpon</u>	107
5.5 Absorption spectra of segments of 8.5% polyacrylamide gels (stained with 0.1% toluidine blue) on which nucleic acid-polyP phenol-detergent extracts had been run	108
5.6 Scans of nucleic acid-polyP phenol-detergent extracts separated on 8.5% polyacrylamide gel and stained with 0.1% toluidine blue	109
5.7 Total P in fractions of 8-d-old mycelia of endophyte of <u>Erica hispidula</u> grown on basal medium containing either 3.23 mM or 0.16 mM $\text{KH}_2\text{PO}_4$ , then incubated for 4 d in basal medium without P	113



5.8 Levels of $^{32}\text{P}$ in P fractions of 7-d-old mycelia of the endophyte of <u>Erica hispidula</u> incubated with $^{32}\text{P}$ and various concentrations of orthophosphate	117
5.9 Percentage of $^{32}\text{P}$ incorporated into P fractions of 7-d-old mycelia of the endophyte of <u>Erica hispidula</u> incubated with $^{32}\text{P}$ and various concentrations of orthophosphate	118
5.10 $^{32}\text{P}$ in fractions of 8-d-old mycelia of the endophyte of <u>Erica hispidula</u> grown on basal medium with either 3.23 mM or 0.16 mM $\text{KH}_2\text{PO}_4$ , then incubated for 4 d in fresh basal medium with $^{32}\text{P}$ orthophosphate	120

## CHAPTER 1

### INTRODUCTION

Heathlands are found in a broad range of climatic zones, stretching from the Tundra and cool climatic regions of the northern hemisphere to the warm and seasonally arid heaths in the mediterranean climatic regions of South Africa and Australia. Despite these variations in climate and geography, heaths are generally characterised by their evergreen, sclerophyllous vegetation restricted mainly to acidic soils of a low nutrient status (Specht, 1979). Many of these heath plants belong to closely related families of the Grubbiaceae, Empetraceae, Pyrolaceae, Epacridaceae and Ericaceae in the Ericales. The heaths of South Africa may be dominated by Erica spp. belonging to the Ericaceae whereas the South Australian heaths contain members of the Epacridaceae.

Nutrient cycling studies in heathlands have concentrated on nitrogen (N) and phosphorus (P) because of their high level of interaction in nutrition and their importance in the control of nutrient cycling processes (Groves, 1983). In South Africa, heathlands form part of the Cape Floral Kingdom (Takhtajan, 1969; Good, 1974) known locally as 'fynbos' (defined by Moll and Jarman, 1984). Mountain fynbos is found exclusively on highly leached acid soils with low nutrient status whereas coastal fynbos often occurs

on well-drained sands of aeolian origin with high base-saturation levels or on lowland limestone (Kruger, 1979). Mitchell, Brown and Jongens-Roberts (1984) showed that coastal fynbos soils of the Clovelly form are very low in total P with the major inorganic form being iron-bound and a considerable proportion as organic P (27 - 60%) These soils are also low in both total and available N and ammonium ( $\text{NH}_4^+$ ) predominates over nitrate ( $\text{NO}_3^-$ ) especially in the later stages of succession of the plant community (Stock and Lewis, in press). In South Australia, levels of soil P and N and organic matter are comparable to those found in the fynbos (Read and Mitchell, 1983). Levels of organic matter and soil P and N in Calluna heathlands in Britain are generally higher than those of the southern hemisphere heathlands (Read and Mitchell, 1983) but nutrient levels may also drop to low levels similar to those of Australia and South Africa (Gimingham, 1972).

The characteristic infertility (or seasonal unavailability of nutrients) in heathland soils and the universal presence of ericaceous plants on them are features accompanied by root systems which are strikingly uniform in structure. The ultimate, fine, hair roots consist of a central stele surrounded by one to three rows of cortical cells (Read, 1983) which may be characteristically infected with endomycorrhizal fungi in an association known as 'ericoid' (Harley, 1969). Under the light microscope, the hair roots

may be commonly surrounded with a loose web of septate mycelium which penetrates the cortical cell walls at points and within the cell proliferates to form dense, hyphal coils (Harley and Smith, 1983; Read, 1983). These features have been observed most commonly in the sub-families Vaccinioideae, Rhododendroideae and Ericoideae of the Ericaceae. Other members of the Ericaceae (Arbutus, Arctostaphylos) form mycorrhizas of the ecto-endo type (arbutoid) as do the Pyrolaceae (Read, 1983) while the Monotropaceae are characterised by a distinctive association called monotropoid (Duddridge and Read, 1982a). Members of the Epacridaceae also form mycorrhizas of the ericoid type (McLennan, 1935; McNabb, 1961).

In recent years, ultrastructural analyses of ericoid mycorrhizas have enhanced our knowledge of the biological nature of the symbiosis and its nutritional and ecological significance. Intracellular hyphae show little evidence of branching, resemble each other morphologically and have simple septa and Woronin bodies characteristic of ascomycetous fungi. The host plasmalemma invaginates to surround individual, penetrating hyphae and is separated from them by a fibrillar, interfacial matrix of varying thickness (Bonfante-Fasola and Gianinazzi-Pearson, 1979; Peterson, Mueller and Englander, 1980; Bonfante-Fasola, Berta and Gianinazzi-Pearson, 1981). Duddridge and Read (1982b) followed the sequence of events involved in the

initiation, establishment and degeneration of the ericoid mycorrhizas of Rhododendron ponticum L., both under natural and sterile conditions and observed that the host cytoplasm degenerates before that of the fungus. As the functional life of the association within a cell lasted no more than seven weeks, it was suggested that if major exchanges of nutrients between fungus and host occur, they would have to take place within a relatively short period. In axenic culture, the infection cycle is even more short-lived with an association of intense metabolic activity (Bonfante-Fasola and Gianinazzi-Pearson, 1982). In Vaccinium myrtillus L. (which is deciduous) infection levels vary throughout the year with peaks in late summer and autumn while in Calluna vulgaris (L.) Hull (an evergreen plant) infection levels are sustained throughout winter but reach a peak in spring when new hair roots form (Bonfante-Fasola, Berta and Gianinazzi-Pearson, 1981). In the seasonally-arid heathlands of the Western Cape however, hair roots are moribund with little or no infection during the dry summer season but are growing actively and highly infected in spring when soil moisture levels are higher (Read, 1978).

It appears that the amounts of mineral P and N which enter heathland ecosystems are low and that the major reserve of these elements is in the soil organic matter (Groves, 1983; Read and Mitchell, 1983). The sclerophyllous vegetation of heathlands can contain high levels of lignin and phenolics

which render the litter resistant to microbial degradation. Microbial activity is further inhibited by the high C : N ratios of the fallen litter and the low mobility of leaf N may also contribute to slow rates of litter decomposition. In Australia and South Africa, the mediterranean-type climate causes seasonal moisture stress which may reduce leaching of minerals from fallen litter, retard microbial decomposition and promote seasonal peaks in mineralising activity. In general, mineralising rates of P and N in heathland ecosystems are low (Read and Mitchell, 1983; Harley and Smith, 1983; Stock and Lewis, in press; Stock, Lewis and Allsopp, in press). Ultrastructural studies of ericoid mycorrhizas suggest that the host root systems have an obligatory requirement for infection by the endophyte which has probably evolved in response to low nutrients. Phenology, climate and features of decomposition processes would determine periods of peak activity of the association when the absorption and exchange of nutrients is maximal and the association of most benefit to both partners.

Ultrastructural investigations have substantiated information on the nutritional and biochemical nature of the ericoid mycorrhizal symbiosis obtained from physiological studies. There is strong evidence (Stribley and Read, 1974a; Pearson and Read, 1973b; Harley and Smith, 1983) to indicate that carbon products flow from autotroph to heterotroph and since the endophytes possess only a limited cellulolytic

ability (Pearson and Read, 1975; Mitchell and Read, 1985) the mycobiont is probably dependent on its host for the bulk of its carbon supply. However, the flow of nutrients from fungus to host is of greater ecological importance, especially with respect to N and P.

### 1.1 NITROGEN NUTRITION

In a typical acid, mor-humus soil of a mature European heathland  $\text{NH}_4^+$  predominates over  $\text{NO}_3^-$  and only 0.4% of the total N is present as free  $\text{NH}_4^+$  while 71% is in the form of hydrolysable organic N (Stribley and Read, 1980). These limiting soil N conditions have stimulated research into the N nutrition of ericoid mycorrhizas, recently reviewed by Harley and Smith (1983) and Read (1983).

It appears from these studies that the mycobiont is able to enhance the N nutrition of its host in two important ways. Firstly, sand-culture studies indicated that the extramatrical hyphae directly aid the uptake of  $\text{NH}_4^+$  ions by their ability to cross depletion zones around the roots and explore a greater volume of soil (Stribley and Read, 1974b; 1976). This is a facility which is probably enhanced by the possible high affinity of fungal absorption systems for  $\text{NH}_4^+$  ions (Harley and Smith, 1983). Secondly, the mycorrhizal root systems appear to have access to sources of N unavailable to non-mycorrhizal roots. Stribley and Read (1980) established that mycorrhizal plants of *V. macrocarpon*

Aiton can utilize amino-acids as sole N sources, an ability which was less developed in aseptically-grown plants or plants infected with common soil saprotrophic fungi. Read (1978) has also suggested that under the conditions of winter rainfall and summer drought experienced in the southern heathlands of the Cape, the endophyte absorbs N efficiently at periods when soil moisture levels are high, and N stored in intracellular hyphal complexes is made available to the host during the critical periods of flowering and seed production. However, in view of the unlikelihood of digestion of the endophyte by the host (Duddridge and Read, 1982b) and the lack of any empirical substantiation, this hypothesis should be treated as speculation.

## 1.2 PHOSPHORUS NUTRITION

Phosphorus is also very limiting in heathland ecosystems and there is no reason to suppose that the endophyte's ability to increase the supply of N to the host does not extend to other elements as well. Moreover, vesicular-arbuscular (VA) endomycorrhizas and ectomycorrhizas contribute to increased growth in their hosts by way of supplementing the supply of P to them (see Harley and Smith, 1983). It seems unlikely that ericoid endophytes do not contribute to their hosts' survival in a similar way. The work on N nutrition of ericoid mycorrhizas and the studies on P nutrition in other



mycorrhizal systems emphasize four main areas of potential research into ericoid mycorrhizal associations.

- (1) Does the endophyte have access to forms of P which the host roots do not?
- (2) Does the endophyte possess more efficient mechanisms for locating and absorbing P than the host roots?
- (3) Is the endophyte able to store P which is later transferred to the host?
- (4) What are the transfer mechanisms involved in the movement of P from fungus to host?

The remainder of this review reflects the field of information which has motivated this project. The field includes studies on VA and ectomycorrhizas as well as investigations into individual aspects of the physiology of other soil fungi. The review article of Beever and Burns (1980) suggests that aspects of the P physiology of soil fungi, including those which form mycorrhizas, are similar. Empirical evidence from studies on other soil fungi may therefore pertain to investigations into the P nutrition of ericoid mycorrhizal endophytes. Since the question of transfer of P at the host/fungus interface was not

approached in this study it will be discussed only in passing.

#### 1.2.1 Access to unavailable sources of Phosphorus

The idea that mycorrhizas are able to tap bound sources of P unavailable to plant roots has been an appealing one for some time; this facility would depend upon the microorganism's accessibility to external substrates by way of active enzyme systems. The low orthophosphate ( $P_i$ ) availability and high organic P levels (especially soluble and insoluble inositol phosphates) in northern hemisphere heathland soils is now well documented (Anderson, 1967; Cosgrave, 1967; Martin and Cartwright, 1971; Anderson, Williams and Moir, 1974; Cheshire and Anderson, 1975; Tinker, 1975). Acid phosphatase (E.C. 3.1.3.2.), an enzyme of broad specificity has been implicated in the process of making P available from organic soil molecules. Other areas of interest are the responses of acid phosphatase activity to high and low P conditions and the localization of activity in the cell under different P conditions.

In ectomycorrhizas, an active acid phosphatase localised in the plasmalemma and cell walls of the sheath of beech roots was able to hydrolyse a wide range of organic esters and inorganic P substrates (Woolhouse, 1969; Bartlett and Lewis, 1973; Williamson and Alexander, 1975). Beech mycorrhizas are also able to use sodium and calcium

phytates in pure culture (Theodorou, 1968; 1971). Ho and Zak (1979) have found large differences in the acid phosphatase activity of six ectomycorrhizal fungi. Alexander and Hardy (1981) found the acid phosphatase activity of mycorrhizal roots of Sitka spruce from a P-deficient site to be inversely proportional to the litter P concentration, a fact which suggested a derepression of phosphatase activity under low Pi conditions. When fractions of three ectomycorrhizal isolates were compared for phosphatase activity under low and high Pi conditions, a large increase in total phosphatase activity (localised mainly in the cell wall) appeared when Pi was deficient (Calleja et al., 1980). Under these conditions, an active extracellular phosphatase was also secreted into the external medium accompanied by a decline in the activity of the soluble (cytoplasmic) enzyme fraction. Calleja and D'Auzac (1983) confirmed these findings and attempted to separate the ectomycorrhizal isolates from three saprotrophic fungi on the basis of their comparative phosphatase activities, but were unable to establish satisfactory parameters for discrimination. Although these experiments demonstrated for the first time that mycorrhizal fungi are able to secrete an extracellular phosphatase, this is not unknown in other fungi (San Blas and Cunningham, 1974; Arnold and Garrison, 1979; Beever and Burns, 1980; Bojović-cvetić and Vujičić, 1982).

Detailed information on the phosphatases of endomycorrhizas is limited to VA mycorrhizas. The levels of soluble acid phosphatase activity of onion roots were not significantly increased by infection with VA endophytes or by the addition of soluble P to the soil (Gianinazzi-Pearson and Gianinazzi, 1976). In a cytochemical study of infected onion roots significant acid phosphatase activity was only observed in immature, terminal arbuscles of the endophyte while alkaline phosphatase activity, which could be repressed at  $P_i$  levels above  $10^{-4}$  M was detected in mature arbuscles and intercellular hyphae (Gianinazzi-Pearson and Gianinazzi, 1978; Gianinazzi, Gianinazzi-Pearson and Dexheimer, 1979). It appears that the differences in activity of the phosphatases was a function of the age of the endophyte and that in mature arbuscles the alkaline phosphatases may be involved in polyphosphate (polyP) metabolism and P transfer to the host.

Pearson and Read (1975) have detected acid phosphatase activity which is inhibited by high concentrations of external  $P_i$  in the ericoid endophyte Pezizella ericae Read. In addition, the endophytes of R. ponticum and V. macrocarpon are capable of using soluble and insoluble phytate salts in culture (Mitchell and Read, 1981). However, whether this utilization is made possible through the mediation of surface-bound or extracellular acid phosphatase is unknown.

One cannot assume that the localisation, function and level of activity of acid phosphatases in all mycorrhizas is going to be similar, especially when these parameters are strongly influenced by experimental conditions. However, in principal, the synthesis of active wall-bound and extracellular phosphatases under conditions of P deficiency may well give mycorrhizal fungi greater access to bound soil P complexes, than other soil saprotrophs or the host itself. Whether these enzymes under cultural conditions are synthesized isoenzymes of functional significance or the products of cell lysis needs to be investigated.

### 1.2.2 Phosphate Absorption

#### Soil Exploration

Free orthophosphate ions, being bound in soil complexes, may be very low in soil solutions and the slow diffusion rates of the ions accompanied by rapid absorption of them by roots leads to the formation of depletion zones around roots (Nye and Tinker, 1977). Harley and Smith (1983) reported that inflow of  $P_i$  (expressed as  $\text{mol cm}^{-3} \text{ s}^{-1}$ ) into onion and clover roots infected with VA endophytes is on the average 3 to 4 times greater than into uninfected controls. The ability of the extramatrical hyphae to continually colonise beyond depletion zones is proposed as the main reason for this greater inflow. A more rapid translocation of  $P_i$  along hyphae than diffusion of  $P_i$  through depletion zones

also contributes to making the mycobiont an effective physical extension of the root system. Thomas et al. (1982) compared the growth of ectomycorrhizal and non-mycorrhizal tree seedlings on  $^{32}\text{P}$ -labelled soil and found that although mycorrhizal seedlings showed a greater P uptake, the proportion of non-labile P absorbed was less than by non-mycorrhizal seedlings. It appears that mycorrhizal roots are able to tap labile P sources beyond root depletion zones whereas non-mycorrhizal roots start using non-labile sources sooner. The only research into P uptake by ericoid mycorrhizal roots showed that the external mycelium of C. vulgaris and V. oxycoccus L. mycorrhizas is able to absorb and translocate P from a source, across a diffusion barrier and to the host plant which acts as the sink (Pearson and Read, 1973b).

#### Phosphate Uptake

If P uptake rates are measured over a narrow range of external  $\text{Pi}$  concentrations they usually indicate a simple hyperbolic relationship with concentration and can be described by the Michalis-Menten constants of  $V_{max}$  and  $K_m$  (Beever and Burns, 1980). The  $K_m$  values depending on the external  $\text{Pi}$  concentrations used, fall into 1 of 2 ranges, either 1 to 10 or 100 to 1000  $\mu\text{M}$ . This indicates the existence of two separate uptake systems, one with a greater affinity for P (lower  $K_m$ ) than the other. Two such separate systems exist in Neurospora crassa Shear and Dodge.

(Lowendorff, Slayman and Slayman, 1974; Lowendorff, Bazinet and Slayman, 1975). The relationship between the two systems has been clarified by Beever and Burns (1977) and Burns and Beever (1977) by studies in which P uptake was monitored at concentrations which encompassed the ranges of both systems. On a transformation plot, the data could no longer be described by a straight line but by two linear portions joined by a distinct curve. Moreover, the growth and  $P_i$  uptake rates remained constant during exponential growth over the broad concentration range used. These authors interpreted their results in terms of a dual uptake system acting simultaneously across the plasmalemma so that compensating changes in the kinetic constants occurred with changes in  $P_i$  concentration. Many fungi appear to possess such systems (Beever and Burns, 1980).

There is some evidence for the operation of dual uptake systems in mycorrhizal fungi. At selected external  $P_i$  concentrations there was a greater rate of uptake by VA mycorrhizal roots than non-mycorrhizal roots when expressed on a mass basis (Bowen, Bevege and Moss, 1975). Cress, Throneberry and Lindsey (1979) examined in more detail the kinetics of P uptake in mycorrhizal and non-mycorrhizal tomato roots. Initial rates of P absorption reached a maximum over the concentration range used and Hofstee transformation plots fitted the data into two linear phases with the transition occurring between 20  $\mu M$  and 30  $\mu M$

$\text{KH}_2\text{PO}_4$  . The  $K_m$  values for mycorrhizal plants were lower than for uninfected roots for both the high and low concentration ranges with an increase in  $V_{max}$  in the high range. In the low range, the increased uptake was a result of an increased site affinity (lower  $K_m$ ) but at the high range it was due to an increase in  $V_{max}$  (i.e. an increase in number of absorbing sites provided by the mycelium external to the roots) (Cress et al., 1979).

There are no detailed comparative kinetic studies on ectomycorrhizal and non-mycorrhizal roots of the same species although Harley and McCready (1950, 1952) have shown that infected beech mycorrhizas incubated in solutions of specific concentrations of external  $\text{Pi}$  absorb five times as much  $\text{P}$  on a surface area basis and twice as much on a mass basis than uninfected roots. Beever and Burns (1980) have compared the kinetic constants of ecto- and VA endomycorrhizas and some plant roots. It is clear from Table 1.1 that conclusive data on the uptake kinetics of mycorrhizas is scanty. Mycorrhizal fungi may not depend exclusively on the exploration of a large soil volume for increased ion absorption but may also rely on a greater efficiency in uptake at the sites of transfer themselves, especially when the nutrient supply is infrequent or very dilute.



TABLE 1.1

Kinetic Constants of the Phosphate Uptake Systems of Excised Mycorrhizas, Fungi and Excised Plant Roots<sup>a</sup>

	High-affinity system		Low-affinity system		
	Km $\mu\text{M}$	Vmax $\mu\text{mol (g d.w.)}^{-1}\text{min}^{-1}$	Km $\mu\text{M}$	Vmax $\mu\text{mol (g d.w.)}^{-1}\text{min}^{-1}$	Capacity
MYCORRHIZAS					
<u>Fagus sylvatica</u>	6.3	0.013	1190	0.250	0.263
<u>Pinus radiata</u>					
type (1)	6.0	0.002	3300	0.038	0.040
type (2)	3.7	0.003	1040	0.057	0.060
TOMATO	1.6	0.017	35	0.053	0.053
FUNGI					
<u>N.crassa</u> (50 $\mu\text{M}$ germlings)	2.43	2.33	370	6.33	8.66
<u>Aspergillus nidulans</u> (50 $\mu\text{M}$ germlings)	9.36	1.98	559	19.9	21.88
PLANT ROOTS					
Tomato (control for mycorrhizal roots)	3.9	0.017	42	0.042	0.042
Millet	3.5	0.182	960	1.145	1.327
Barley	8.0	0.109	900	0.400	0.509
	5.4	0.100	490	0.273	0.373
Lucerne (alfalfa)	2.7	0.017	940	0.181	0.198
	4.3	0.100	800	0.556	0.656
<u>Stylosanthes humilis</u>	6.1	0.339	303	0.379	0.718

<sup>a</sup> (Beever and Burns, 1980)

### 1.2.3 Phosphate storage - the role of polyphosphates

The pioneering, histochemical staining techniques of Ebel and Muller (1958); Ebel, Colas and Muller (1958a & b); Muller and Ebel (1958) have been used to demonstrate the presence of metachromatic, vacuolar granules in ectomycorrhizas (Ashford, Ling-Lee and Chilvers, 1975) and VA mycorrhizas (Cox et al., 1980). In addition, the transmission electron microscope (TEM) and X-ray microanalysis have shown that these granules in ectomycorrhizas (Strullu et al., 1981; Strullu et al., 1982, 1983) and VA mycorrhizas (White and Brown, 1979; Cox et al., 1980) contain high concentrations of P. The P occurs in the form of inorganic polyphosphate (polyP) chains (Harold, 1966). PolyP granules have in fact been observed in a wide range of fungi (Ebel and Muller, 1958; Ebel et al., 1958b; Muller and Ebel, 1958; Chilvers, Lapeyrie and Douglass, 1985) and may be regarded as representing a common strategy whereby P is stored in a condensed form, especially when freely available (Beever and Burns, 1980). Considerable work is now being done to clarify the characteristics of polyP metabolism in mycorrhizal fungi and hence its role in the P nutrition of the host.

The early  $^{32}\text{P}$  radioactive work of Harley and his associates on P uptake by beech mycorrhizas showed that P reaches the host cells via the fungal protoplast with uptake and

transfer to the host being active processes (see Harley, 1969). The exchanges are mediated by a small pool of  $P_i$  and most  $P$  which enters the fungus is shunted into a large, inorganic pool. When  $P$  is not available from the external environment, it can be mobilised from the large pool to supplement the smaller, labile pool and hence supply the host tissues (Harley, 1969). It became clear later (Chilvers and Harley, 1980) that the site of accumulation of the reserve inorganic  $P$  was in the vacuoles, in the form of poly $P$  granules which accumulated as a result of external  $P$  levels. Lapeyrie, Chilvers and Douglass (1984) have recently observed a similar trend in pure cultures of the ectomycorrhizal fungus, Paxillus involutus (Batsch) Fr..

However, quantification of poly $P$  levels in relation to the levels of other  $P$  pools under controlled conditions requires elaborate fractionation procedures. Harley and McCready (1981) used the fractionation technique of Aitchison and Butt (1973) which is based on the traditional distinction between acid-soluble (short-chain) and acid-insoluble (long-chain) poly $P$  both precipitated by  $BaCl_2$  (See Harold, 1966) to establish that  $^{32}P$  absorbed by Fagus mycorrhizas accumulates initially as  $P_i$  but over 2.5 hours is converted to high levels of poly $P$  compounds (40% of the total absorbed  $^{32}P$ ). In comparison pure cultures of ectomycorrhizal fungi store only low levels of poly $P$  even

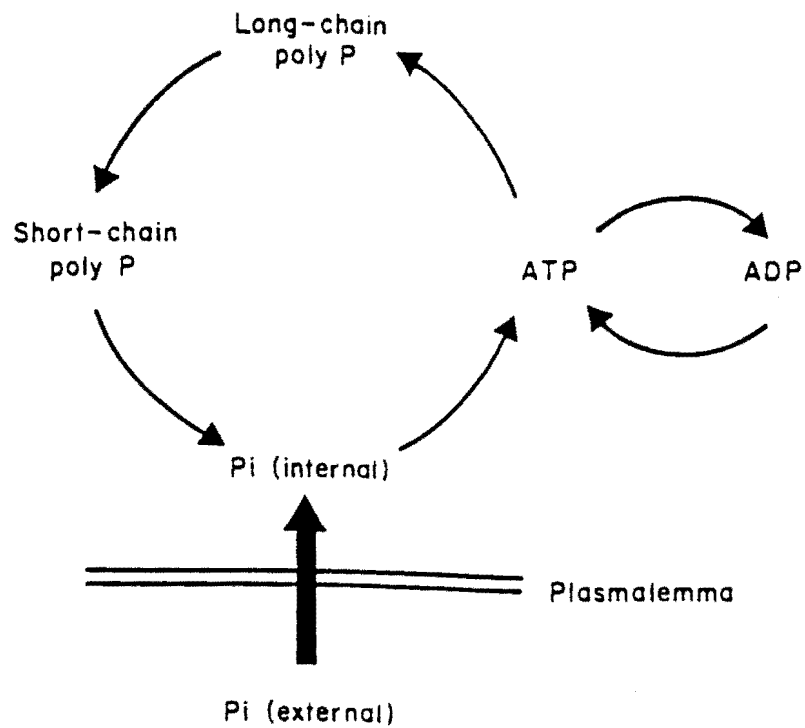
under conditions of P surplus (Martin et al., 1983; Rolin, Le Taçon and Larher, 1984)

Callow et al. (1978), critical of fractionation techniques based on  $\text{BaCl}_2$  precipitation of polyP with its risk of contamination with nucleic acids, employed a method of phenol-detergent extraction of undegraded polyP-nucleic acid co-precipitates which were finally separated by polyacrylamide gel electrophoresis. They estimated a VA endophyte in onion roots to contain 40% of its total P as polyP. Since uninfected roots did not contain polyP and host cells did not stain metachromatically they assumed the polyP to be fungal in origin. Later the polyP content of isolated, intact VA endophytes was measured more accurately to be 16% of the total P (Capaccio and Callow, 1982).

Although polyP might be an important storage form of  $\text{P}_i$  it may also fulfil an intricate function in the overall P metabolism of the fungal cell and be linked to  $\text{P}_i$  transport to the host and sugar transport from the host. Capaccio and Callow (1982) suggest that the polyP cycle in VA mycorrhizas probably resembles that proposed for other microorganisms (Harold, 1966; Beever and Burns, 1980).  $\text{P}_i$  levels in the cell remain relatively constant by virtue of a net synthesis of long-chain polyP via ATP (catalysed by polyP kinase) in times of P excess and a stepwise breakdown of long-chain polyP to shorter chains and eventually  $\text{P}_i$

(catalysed by the polyphosphatases) in times of P deficiency (Fig. 1.1). Hydrolysis of polyP and release of Pi across the tonoplast would maintain a high Pi concentration in the cytoplasm adjacent to the fungal/host interface, facilitating passive movement into the interfacial apoplast for active uptake by the host (Harley and Smith, 1983).

Cappacio and Callow (1982) have shown that extracts of internal VA endophytes and infected onion roots can catalyse the transfer of terminal P from ATP to a high molecular weight form with the characteristics of polyP, a reaction mediated by polyP kinase. PolyP kinase activities were also greatly increased in infected roots when transferred from a solution of  $10^{-5}$  M Pi to  $10^{-3}$  M Pi and a stronger activity of exo- and endopolyphosphatases in infected than uninfected roots was detected (Cappacio and Callow, 1982). In association with sugar uptake, it is proposed that polyP breakdown is linked to hexose uptake into the fungus by the synthesis of hexose-phosphate, catalysed by polyP hexokinase (Woolhouse, 1975; Harley and Smith, 1983). The phosphorylated hexoses could be used in metabolism or the formation of oligo- or polysaccharides and the Pi be released into the labile Pi pool. PolyP hexokinase is active in both internal and external VA hyphae (Cappacio and Callow, 1982). Alternatively, polyP kinase in its reverse reaction could generate ATP at a site of transfer; the ATP



**FIG. 1.1** Diagram of the polyphosphate cycle showing interrelationships between polyphosphate, the adenine nucleotides and orthophosphate (from Beever and Burns, 1980).

would then be used in the active uptake of hexose to form hexose phosphate (Harley and Smith, 1983).

PolyP has been further implicated in the P nutrition of VA mycorrhizas through its prospective role in P translocation (Cox and Tinker, 1976; Cooper and Tinker, 1981; Cox et al, 1980). It is apparent that an elaboration of some or all of the potential functions of the molecule is essential to an investigation into the P nutrition of mycorrhizal fungi.

### 1.3 AIMS AND OBJECTIVES

The objectives of this project were to investigate the phosphorus nutrition of ericoid mycorrhizas with a view to clarifying their importance in the nutrition of their host plants. On the basis of the information reviewed three aspects were selected for investigation:

(1) to determine the activity and character of the acid phosphatases of the endophyte in culture with special interest in an extracellular fraction.

(2) to investigate the P uptake kinetics of a South African endophyte in culture with emphasis on the possible operation of a dual uptake system. This would provide preliminary evidence for indicating the potential of ericoid mycorrhizas to facilitate P uptake under conditions of erratic P supply

(3) to identify, characterise and estimate the levels of polyphosphates in endophytes and synthesized mycorrhizal seedlings and establish the importance of the molecule as a P storage form under conditions of excess P availability.



## CHAPTER 2

### MATERIALS AND METHODS

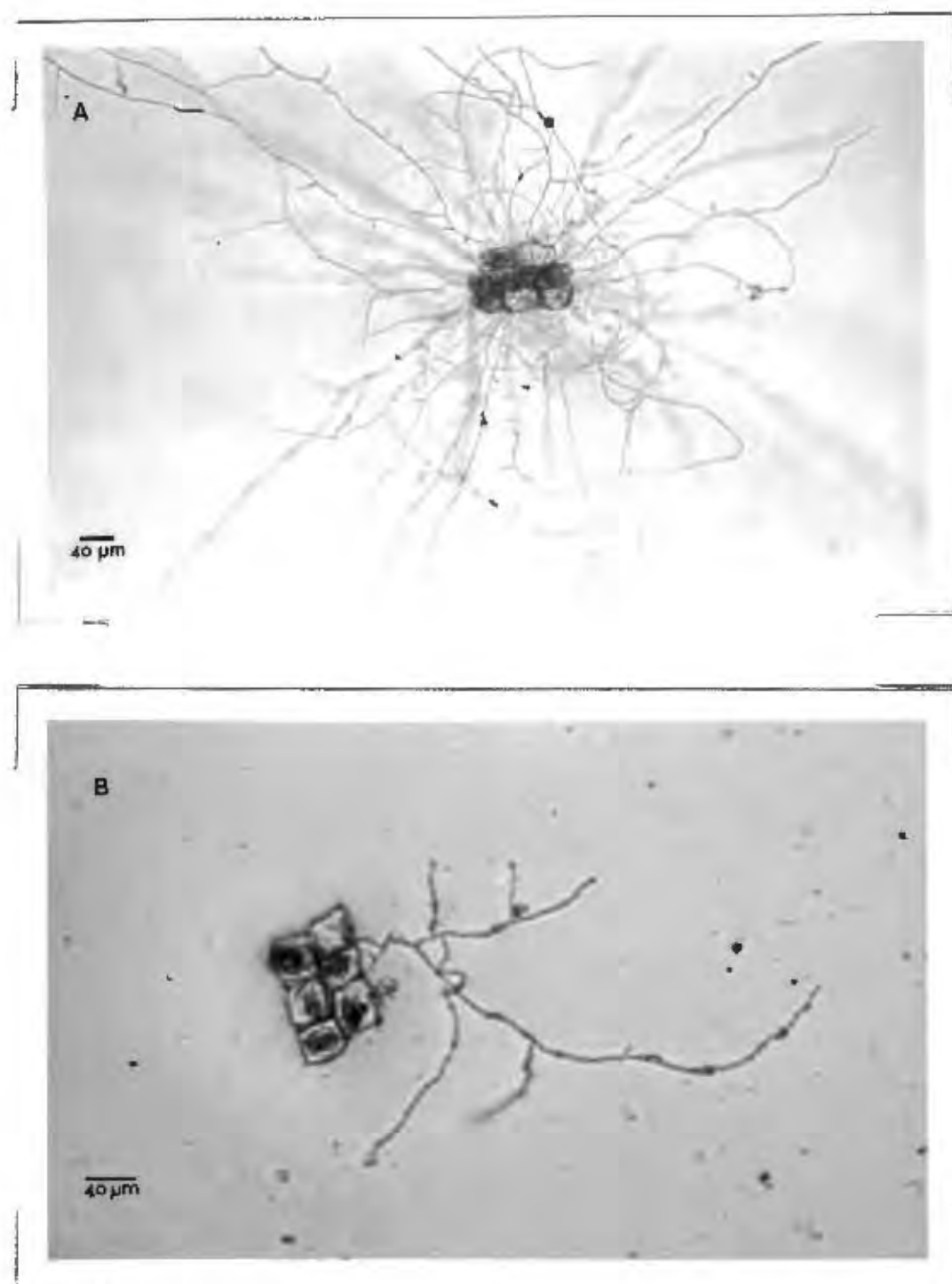
#### 2.1 CULTURAL PROCEDURES

##### 2.1.1 Isolation of endophytes

Mycorrhizal endophytes of V. macrocarpon, R. ponticum and C. vulgaris from the United Kingdom and E. hispidula and E. mauritanica from South Africa were isolated from root systems using the serial washing and maceration techniques described by Pearson and Read (1973a). Cultures of V. macrocarpon and R. ponticum were those used by Mitchell and Read (1981) whereas the endophyte of C. vulgaris was isolated from seedlings taken from Parys Mountain, Anglesey, United Kingdom. Seedlings of E. hispidula and E. mauritanica were growing in acid Table Mountain sandstone soils at the National Botanical Gardens, Kirstenbosch and Tokai forest respectively, 15 to 18 km S.E. of Cape Town. All the endophytes were grown on 2% malt extract agar. The South African isolates were successfully back-inoculated into seedlings of V. macrocarpon and re-isolated (Fig. 2.1).

##### 2.1.2 Synthesis of mycorrhizal root systems

Seeds extracted from fresh fruits of V. macrocarpon were surface sterilized in 3% sodium hypochlorite for 5 min, washed thoroughly with sterile distilled water and



**FIG. 2.1** Cortical cells from root systems of 3-month-old seedlings of *Vaccinium macrocarpon* showing infection by the endophytes of (A) *Erica mauritanica* and (B) *Erica hispidula*.

transferred to sterile plates containing 1% agar. After three weeks, seedlings were transferred to McCartney bottles containing 20 cm<sup>3</sup> of the following autoclaved medium (Robbins and White, 1936): MgSO<sub>4</sub>.7H<sub>2</sub>O, 10 mg; KH<sub>2</sub>PO<sub>4</sub>, 10 mg; FeCl<sub>3</sub>. 6H<sub>2</sub>O, 2 mg; NH<sub>4</sub>Cl, 32 mg; CaCl<sub>2</sub>. 6H<sub>2</sub>O, 33.5 mg; agar, 10.0 g with distilled water to 1 dm<sup>3</sup>, supplemented with 0.5 g dm<sup>-3</sup> glucose, 1 g dm<sup>-3</sup> activated charcoal (Duclos and Fortin, 1983) and covered with a thin layer of autoclaved acid-washed sand when set. The uncapped bottles were placed in autoclaved glass boxes (47 cm x 32.6 cm x 20 cm high) standing in stainless steel trays, which were placed in growth cabinets with 16 h daylight at 20 °C and 8 h darkness at 15 °C and an irradiance of 30 W m<sup>-2</sup>.

After six weeks, the lightly infected seedlings were transferred to moist, sterile Clovelly soil and grown for another six weeks under the same conditions by which time infection of the root system was sufficiently developed to permit harvesting of the seedlings. All root systems were thoroughly washed under tap water and in a number of changes of distilled water prior to either extraction and digestion procedures or re-isolation of the endophytes.

### 2.1.3 Preparation of liquid cultures

The basal liquid nutrient medium used for all experiments was similar to that used by Mitchell and Read (1981) and

fraction. These fractions were assayed for phosphatase activity and total protein. Further purification and characterisation of these fractions were undertaken using methods in sections 2.2.2 and 2.2.3.

#### 2.2.2 Procedure for the fractionation of mycelia of the endophyte of *E. hispidula* prior to partial purification and characterisation of acid phosphatase

The extraction and fractionation procedure was based upon that of Hasegawa, Lynn and Brockbank (1976).

##### Extracellular fraction

Cultures of the endophyte of *E. hispidula*, growing on 3.23 mM sodium inositol hexakisphosphate were harvested at 13 days, i.e. during the exponential growth phase (Fig. 3.1). Each mycelium, filtered under suction, was rinsed in 10 cm<sup>3</sup> ice-cold, distilled water. The filtered liquid media of 20 cultures together with the washings were passed through a "Millipore" filter (0.45  $\mu$ M), dialysed against distilled water at 10 °C, lyophilised in a New Brunswick freeze-drier and dissolved in 20 cm<sup>3</sup> 0.1 M acetate buffer, (pH 4.5) to form the crude extracellular fraction.

##### Cytoplasmic fraction

Mycelia were bulked, thoroughly washed in ice-cold, distilled water, homogenised in distilled water and centrifuged twice at 12 000 x *g* for 15 min. The supernatant was dialysed

against distilled water, concentrated by lyophilisation and dissolved in 20 cm<sup>3</sup> 0.1 M acetate buffer (pH 4.5) to form a crude cytoplasmic fraction.

#### Wall- and membrane-bound fractions

The residue was suspended in cold 0.2% Triton X-100 solution (a non-ionic detergent which dislodges cytoplasmic enzymes attached to cell membranes) for 2 h, strained through a number of layers of muslin cloth and washed repeatedly with Triton X-100 solution until negligible phosphatase activity was detected in the washing medium. The Triton X-100 filtrate was dialysed against distilled water, concentrated by lyophilisation and dissolved in 0.1 M acetate buffer (pH 4.5) to form a membrane-bound fraction. The residue was rinsed with distilled water and incubated in 1 M NaCl at 0 °C overnight to release wall-bound proteins (see Fig. 3.2). The suspension was centrifuged at 12 000 x g for 15 min, the supernatant dialysed against distilled water, lyophilised and dissolved in 20 cm<sup>3</sup> 0.1 M acetate buffer (pH 4.5) to give a soluble, wall-bound fraction. The residue debris formed the insoluble, wall-bound fraction.

#### 2.2.3 Gel filtration

The acid phosphatase activity of the crude enzyme fractions was determined immediately (section 2.1.4) and then stored at -20 °C for a maximum of two weeks. The crude enzymes were further purified by eluting 3 cm<sup>3</sup> volumes

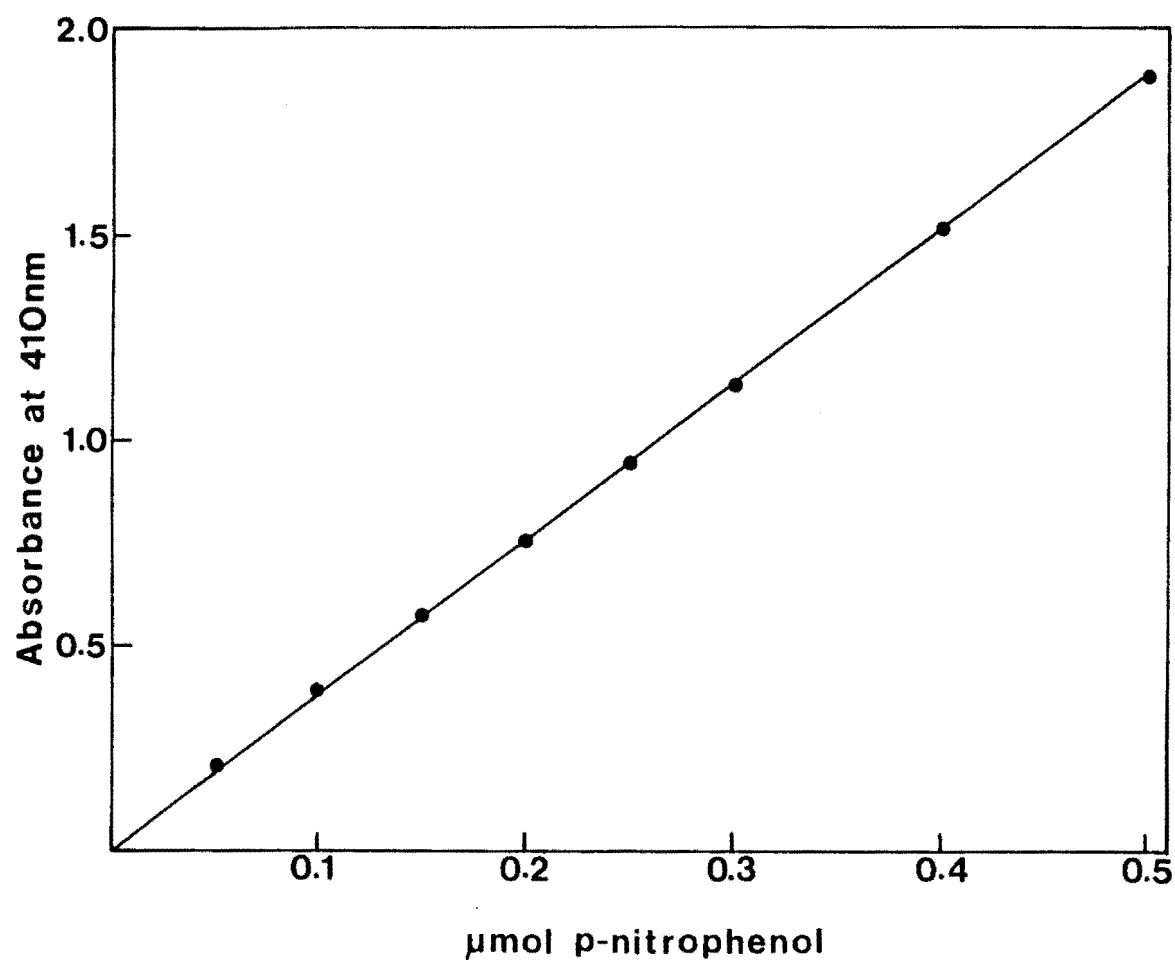


FIG. 2.2 Standard curve showing the relationship between absorbance at 410 nm and amount of p-nitrophenol in the incubation medium. The relationship is  $y = 3.75x + 0.02$  ( $r = 0.94$ ) and each point represents a mean of 3 replicates.

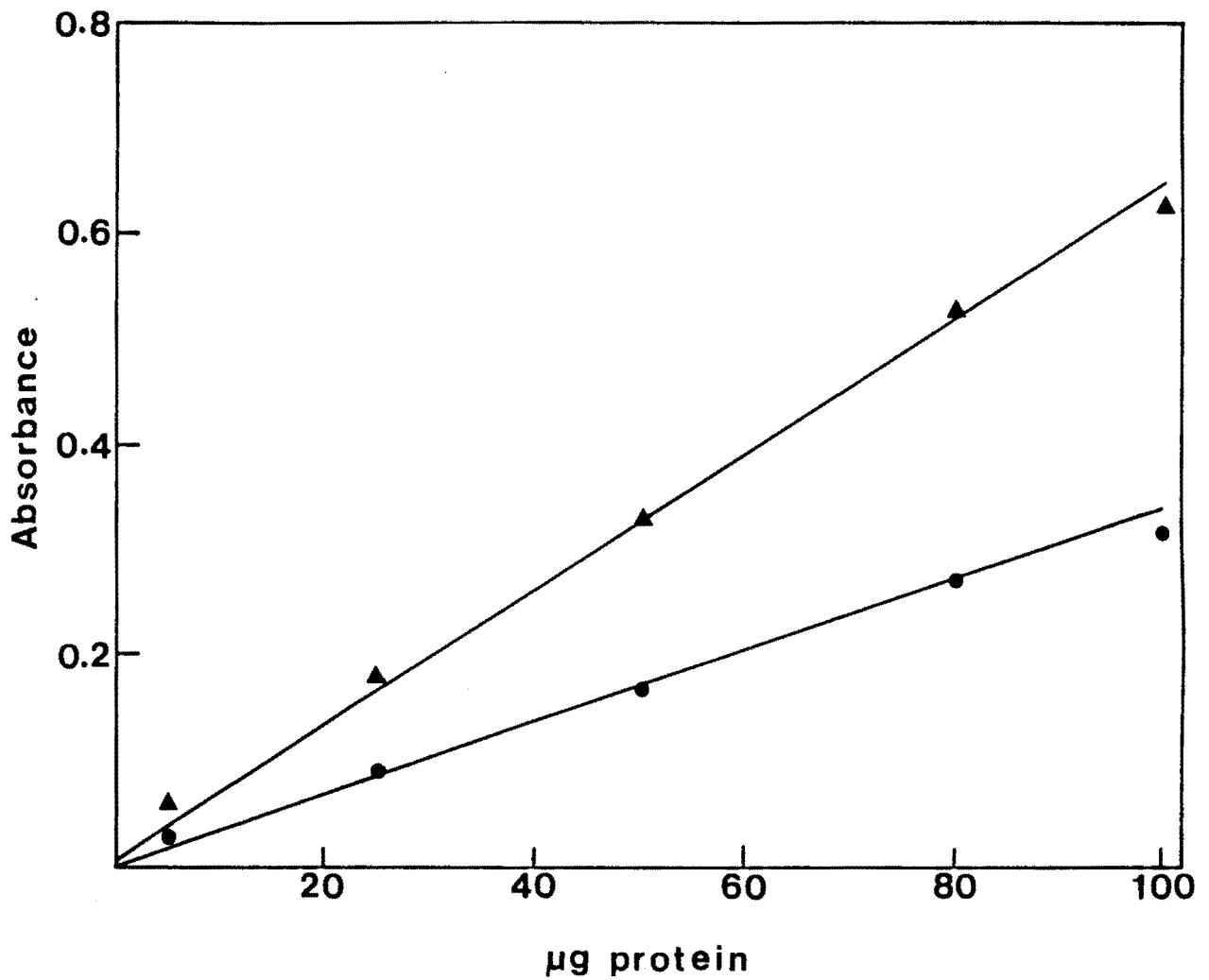
M NaOH (Bartlett and Lewis, 1973). Absorbance at 410 nm was measured on a Pye Unicam SP 1800 spectrophotometer and converted to units of  $\mu\text{mol}$  PNP released (Fig. 2.2). A control without the enzyme was always included to measure non-enzymatic hydrolysis of the substrate. The incubation medium used to measure the effects of different compounds on phosphatase activity contained  $0.1 \text{ cm}^3$  enzyme extract,  $0.9 \text{ cm}^3$  of the appropriate buffer (determined from pH scans) in which was dissolved the effector compound and  $1 \text{ cm}^3$  PNPP as substrate. Controls without the enzymes were run to adjust the absorbance readings at 410 nm for colour contamination.

#### 2.2.5 Protein determinations

Total protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard (Fig. 2.3).

#### 2.2.6 Phosphorus assay

The affinity of the enzyme for different substrates was determined by measuring the level of orthophosphate released into the incubation medium ( $0.1 \text{ cm}^3$  enzyme extract,  $0.9 \text{ cm}^3$  buffer,  $1 \text{ cm}^3$  5 mM substrate) after 30 min incubation at  $25^\circ\text{C}$  in a shaking water-bath. When sodium phytate was the substrate, incubation occurred for 24 h. The final substrate concentration of 2.5 mM was equivalent to the concentration of PNPP under standard conditions. Phosphate was assayed by



**FIG 2.3** Standard curves showing the relationship between absorbance at two wavelengths and protein (bovine albumen) content in the incubation medium, assayed by the method of Lowry et al. (1951). (●—●, 500 nm; ▲—▲ 750 nm). The relationship is  $y = 0.003x + 0.01$  ( $r = 0.99$ ) at 500 nm and  $y = 0.006x + 0.03$  ( $r = 0.99$ ) at 750 nm. Data points represent the means of 3 replicates.



the method of Murphy and Riley (1962) (Fig. 2.4) and controls without the enzymes were run to measure non-enzymatic hydrolysis or free orthophosphate contamination of the substrates. All substrates were obtained from either Sigma or Boehringer Mannheim.

#### 2.2.7 Gel electrophoresis

Electrophoresis was initially performed according to Davis (1964) with the use of vertical, flat-bed gels of 5%, 7.5% or 10% polyacrylamide. No migration of the proteins occurred either under basic (0.05 M tris-glycine running buffer, pH 8.3) or acidic (0.05 M  $\beta$ -alanine-acetic acid running buffer, pH 4.5) conditions during 24 h incubation at 30 mA constant current. The enzymes finally migrated after more than 24 h at 30 mA constant current in horizontal flat-beds containing 0.5% agarose with a 0.1 M tris-acetate-EDTA running buffer adjusted to pH 4.4 with acetic acid. Acid phosphatase was identified by incubating the gels for 2 h at room temperature in 250 cm<sup>3</sup> 0.2 M acetate buffer (pH 4.0), containing 250 mg  $\beta$ -naphthylphosphate and 250 mg fast garnet GBC salt.

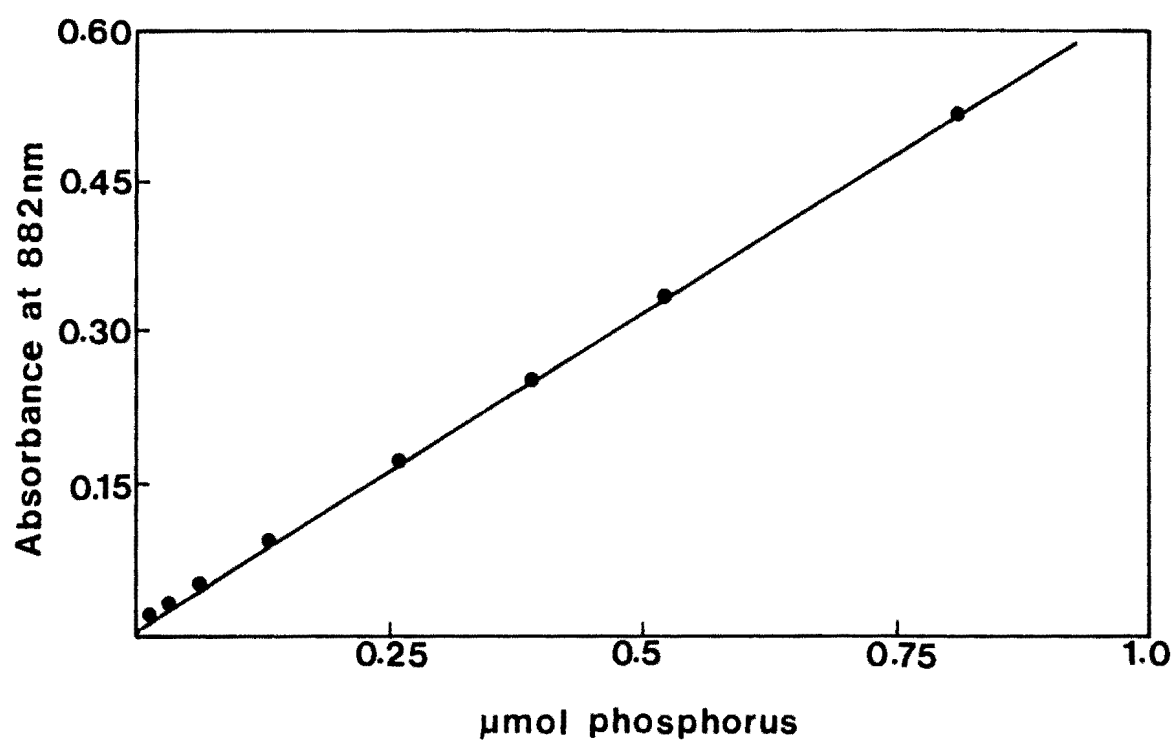


FIG. 2.4 Standard curve showing the relationship between absorbance at 882 nm and level of P using  $\text{KH}_2\text{PO}_4$  and the method of Murphy and Riley (1962). The relationship is  $y = 0.02x + 0.01$  ( $r = 0.99$ ) and data points are the means of 3 replicates.

## 2.3 THE KINETICS OF PHOSPHATE UPTAKE BY THE ENDOPHYTE ISOLATED FROM ROOT SYSTEMS OF ERICA HISPIDULA

### 2.3.1 General incubation and radioisotope counting procedures

Active mycelia, growing on the basal liquid medium (Section 2.1.3) were isolated during the logarithmic phase of growth, washed in ice-cold, distilled water, blotted dry and incubated for 15 min in 3 cm<sup>3</sup> 0.5 mM CaSO<sub>4</sub> at room temperature. The incubation in CaSO<sub>4</sub> is supposed to increase membrane permeability and reduce efflux of absorbed <sup>32</sup>P isotope (Jennings, 1964; Harrison and Helliwell, 1979). Mycelia were blotted, transferred to 3 cm<sup>3</sup> of the incubation medium and incubated in a shaking water bath at 25 °C. Incubation media consisted of <sup>32</sup>P isotope dissolved in either 0.5 mM CaSO<sub>4</sub> (pH 4.6) or selected buffers. In the experiment to test the effect of pH on uptake kinetics, the pH buffers were 0.1 M acetate (pH 4.6 and 5.6), 0.1 M maleate (pH 6.8) and 0.1 M barbital (pH 7.9). The effect of a metabolic inhibitor on uptake was tested by using 0.5 mM 2,4-dinitrophenol in 0.1 M acetate buffer (pH 5.6). Solutions for the experiments to determine the kinetic constants of high-P and low P-fed mycelia were prepared as follows: 500 µCi KH<sub>2</sub><sup>32</sup>PO<sub>4</sub><sup>-3</sup> dm<sup>-3</sup> (equivalent to 1.64 µM) was added to a stock solution of orthophosphate (KH<sub>2</sub>PO<sub>4</sub>) to give a final phosphate concentration of 0.5 mM in 0.1 M acetate buffer (pH 5.6). The stock solution was diluted to give feeding solutions ranging from 1 µM to

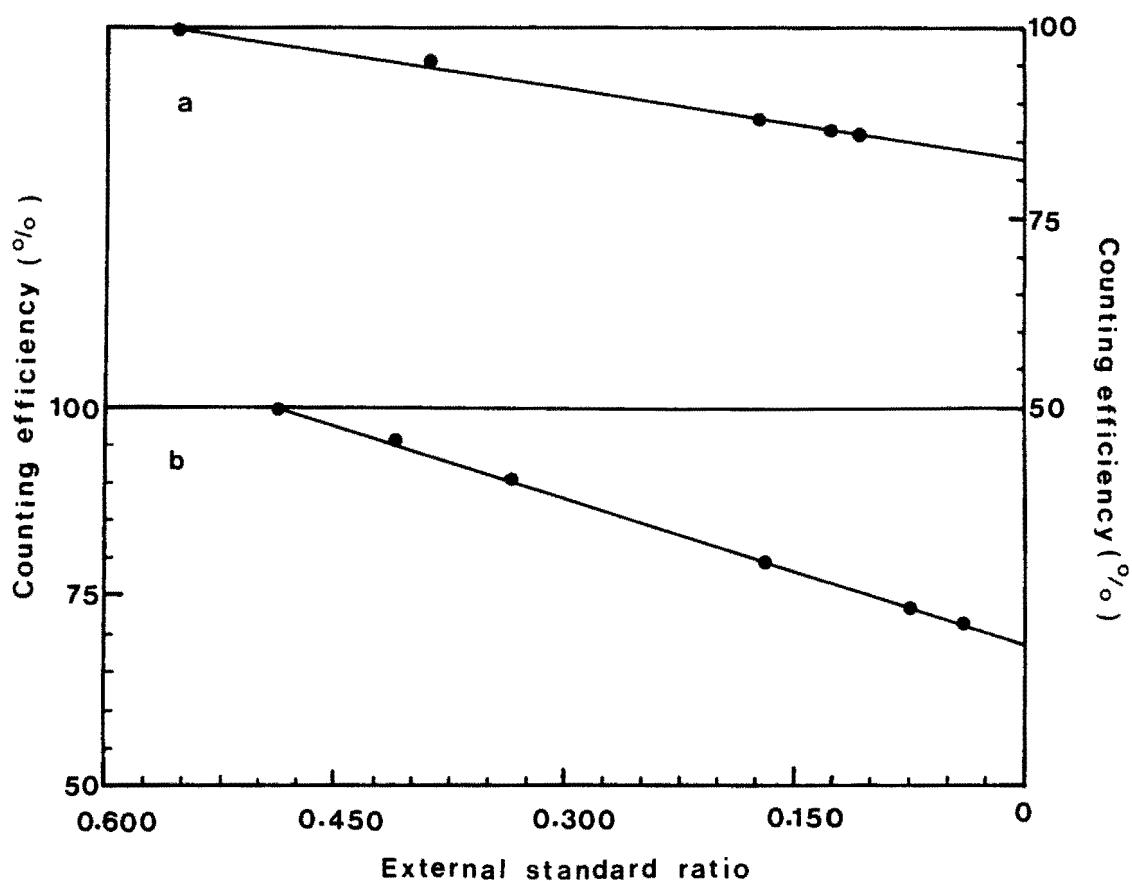
0.5 mM phosphate and  $1 \mu\text{Ci dm}^{-3}$  to  $500 \mu\text{Ci dm}^{-3}$   $^{32}\text{P}$ -ortho-phosphate.

After incubation, the mycelia were removed, washed in  $10 \text{ cm}^3$  distilled water, oven-dried at  $70^\circ\text{C}$  overnight and weighed. Dried mycelia were solubilised in  $0.6 \text{ cm}^3$  of a 40% hydrogen peroxide/60% perchloric acid mixture (in a ratio of 2 : 1) for 2 h after which was added  $10 \text{ cm}^3$  scintillation cocktail containing a chemiluminescence inhibitor (Packard Dimilume-30). Samples were counted for up to 10 min in a Beckman LS-150 liquid scintillation spectrometer at maximum channel window width with automatic quench compensation (AQC) calibrated on an External Standard ratio value of 0.744 with preset error at 0.2%. Percentage counting efficiency was estimated from quenched curves (Fig. 2.5) prepared with hydrogen peroxide/perchloric acid mixtures ( $0$  to  $1.8 \text{ cm}^3$ ) and  $0.05 \mu\text{Ci } ^{32}\text{P}$  and counts in CPM were converted to disintegrations per minute (DPM) by the equation:

$$\text{DPM} = \frac{\text{CPM}}{\% \text{ efficiency}} \cdot 100$$

Counts were also corrected for decay and background activity. Activity (in DPM) was related to the quantity of P absorbed from the incubation solution by means of the equation (Harrison and Helliwell, 1979):

$$Y_2 = A(C/B), \text{ where } Y_2 = \text{uptake of } ^{32}\text{P} \text{ by mycelia (pmol } ^{32}\text{P mg}^{-1} \text{ dry mass unit time}^{-1} \text{ )};$$



**FIG. 2.5** Curves used for the correction of radioactive counts (cpm) from (a)  $\text{KH}_2^{32}\text{PO}_4$  and (b) glucose-6- $^{32}\text{P}$  due to quenching. The curves fitted the straight-line equations,  $y = 32.5x + 83.2$ , with  $r = 0.90$  (a) and  $y = 65.6x + 68.3$ , with  $r = 0.99$  (b). Data points are the means of 3 replicates.

A = amount of  $^{32}\text{P}$  in the feeding solution;

B =  $^{32}\text{P}$  activity (dpm) of the feeding solution and

C =  $^{32}\text{P}$  activity (dpm)  $\text{mg}^{-1}$  dry mass

A value for A was determined from four replicates by the method of Kempers (1975) (Fig. 2.6).

### 2.3.2 Efflux studies

To assess  $^{32}\text{P}$  ion efflux during uptake, mycelia grown on 0.06 mM and 6 mM  $\text{KH}_2\text{PO}_4$  in the basal medium were suspended in a medium containing 0.02  $\mu\text{M}$   $^{32}\text{P}$  (0.06 mM mycelia) or 0.3  $\mu\text{M}$   $^{32}\text{P}$  (6 mM mycelia) for 5 min. Mycelia were harvested, washed in distilled water and resuspended in a medium either in the absence of phosphate or containing non-radioactive P at 0.06 mM, 0.6 mM, 6 mM or 24 mM. The radioactivity of the mycelia was measured at periods up to 15 min for each of the concentrations (Burns and Beever, 1977).

### 2.3.3 Derivation of kinetic parameters

The kinetic parameters of phosphate uptake by the endophyte of E. hispidula were estimated by assuming the simultaneous operation of two uptake systems each obeying Michaelis-Menten kinetics which has been applied to the uptake kinetics of Neurospora crassa (Beever and Burns, 1977; Burns and Beever, 1977).

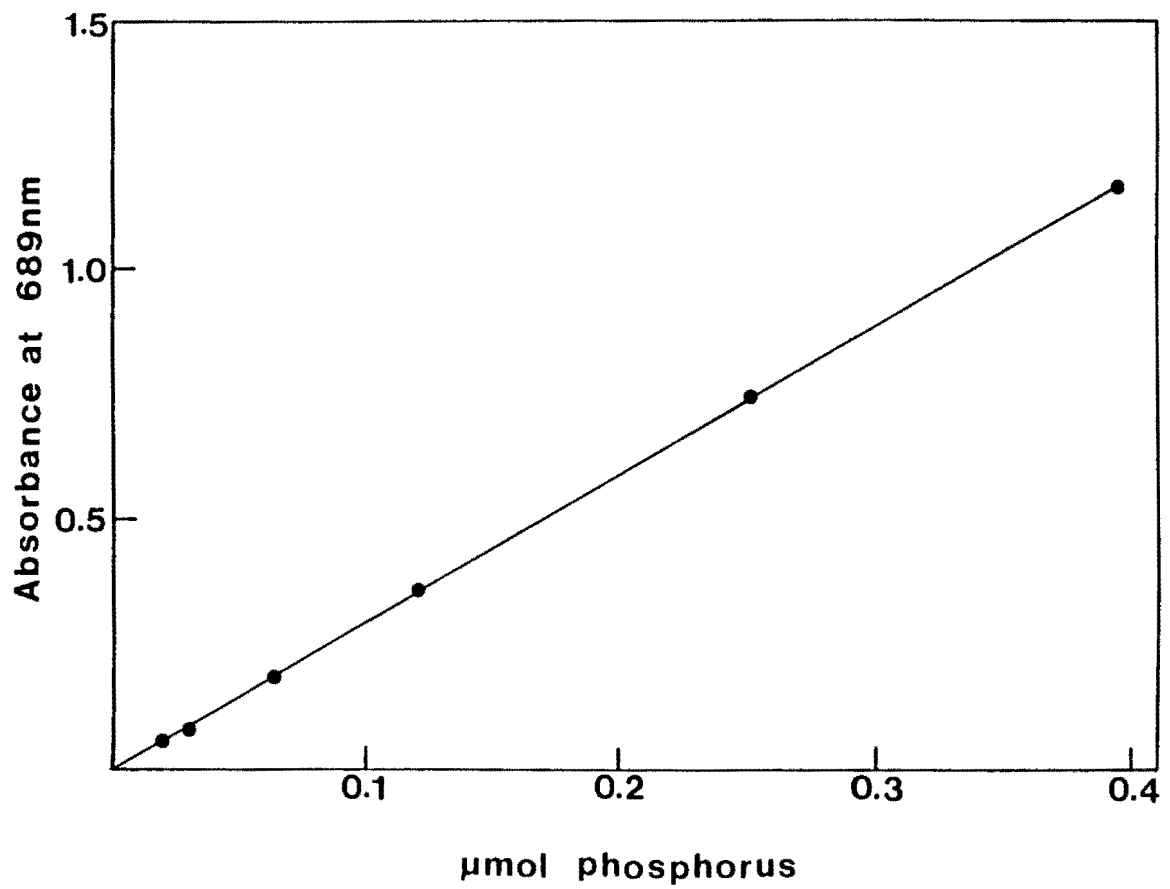
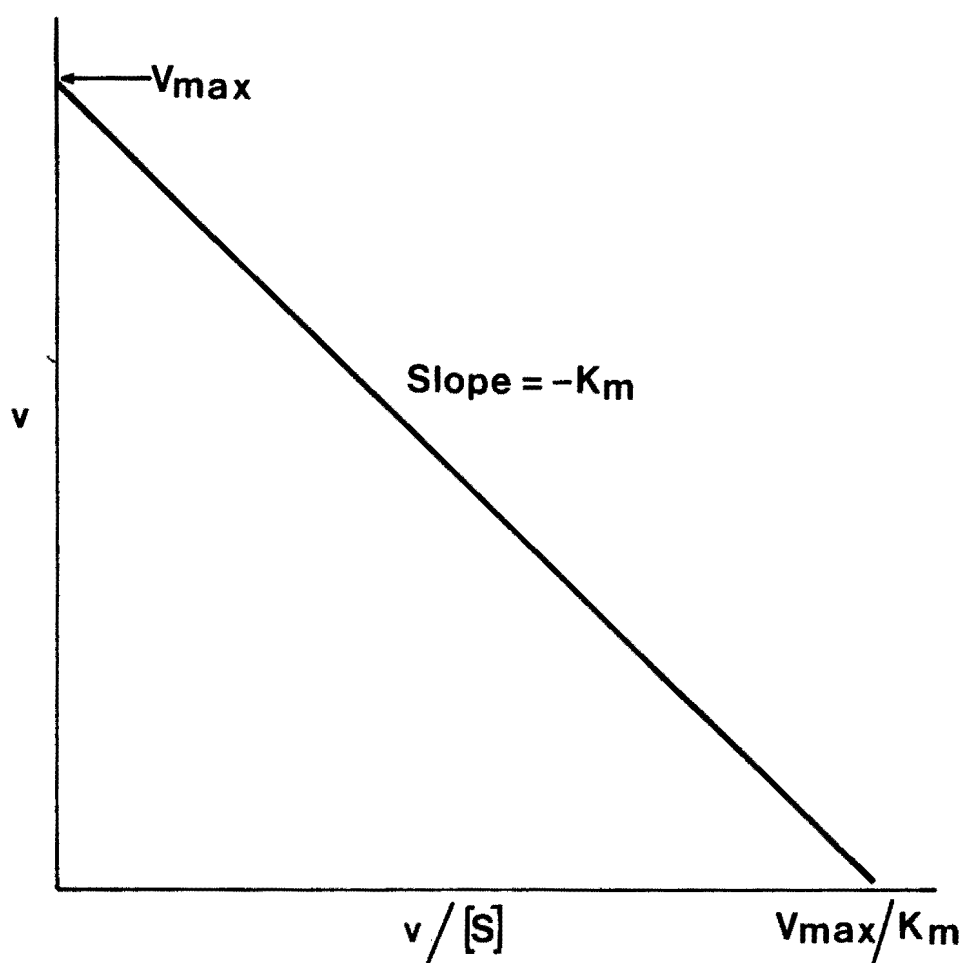


FIG. 2.6 Standard curve showing the relationship between absorbance (O.D.U.) and level of P in the incubation medium, assayed by the method of Kempers (1975). The curve fits the equation  $y = 0.1x - 0.01$ ,  $r = 0.99$  and points are the means of 3 replicates.

$$v = \frac{V_{max}(LA) \cdot S}{K_m(LA) + S} + \frac{V_{max}(HA) \cdot S}{K_m(HA) + S}$$

where  $v$  = uptake rate;  $S$  = phosphate concentration;  $V_{max}(LA)$  and  $V_{max}(HA)$  are the maximum uptake rates of the low-affinity and high-affinity systems, respectively; and  $K_m(LA)$  and  $K_m(HA)$  are the  $P$  concentrations which give rise to half-maximum uptake rates for each system. The kinetic parameters  $V_{max}(LA)$ ,  $V_{max}(HA)$ ,  $K_m(LA)$  and  $K_m(HA)$  were derived from uptake measurements made over a wide  $P$  concentration range (1  $\mu$ M to 0.5 mM) by using a computer-based method to fit the double-hyperbola equation to the data. The programme in BASIC was written by Burns and Tucker (1977) and modified for use on a SPERRY 20 microcomputer (Appendix I). Data were partitioned into two subsets, with each being repetitively solved for a single hyperbola, using the direct linear plot method, after subtracting the calculated contribution of the hyperbola corresponding to the other subset. Direct linear plots, a non-parametric method for the estimation of kinetic parameters (Cornish-Bowden and Eisenthal, 1974; Eisenthal and Cornish-Bowden, 1974) have been adapted and evaluated for use in the fitting of data to a double hyperbola equation (Burns and Tucker, 1977).





**FIG. 2.7** Generalised plot of the Hofstee linear transformation of the Michaelis-Menten equation. The straight line fits the equation  $v = V_{max} - K_m \cdot \frac{v}{[S]}$

$v$  represents rate of reaction or rate of uptake;  $s$  represents substrate concentration;  $V_{max}$  is the maximum rate of reaction and  $K_m$  is the substrate concentration at which  $\frac{1}{2} V_{max}$  occurs. Curvilinear plots are obtained when data can be fitted to a double-hyperbola equation.

The relationship between P concentration and initial uptake rates as calculated by the double-hyperbola curve fit programme is presented in the form of a Hofstee transformation (Hofstee, 1959; Fig. 2.7).

## 2.4 IDENTIFICATION, EXTRACTION AND ESTIMATION OF POLY-PHOSPHATE AND PHYTIC ACID

### 2.4.1 Cytochemical methods for the identification of polyphosphates

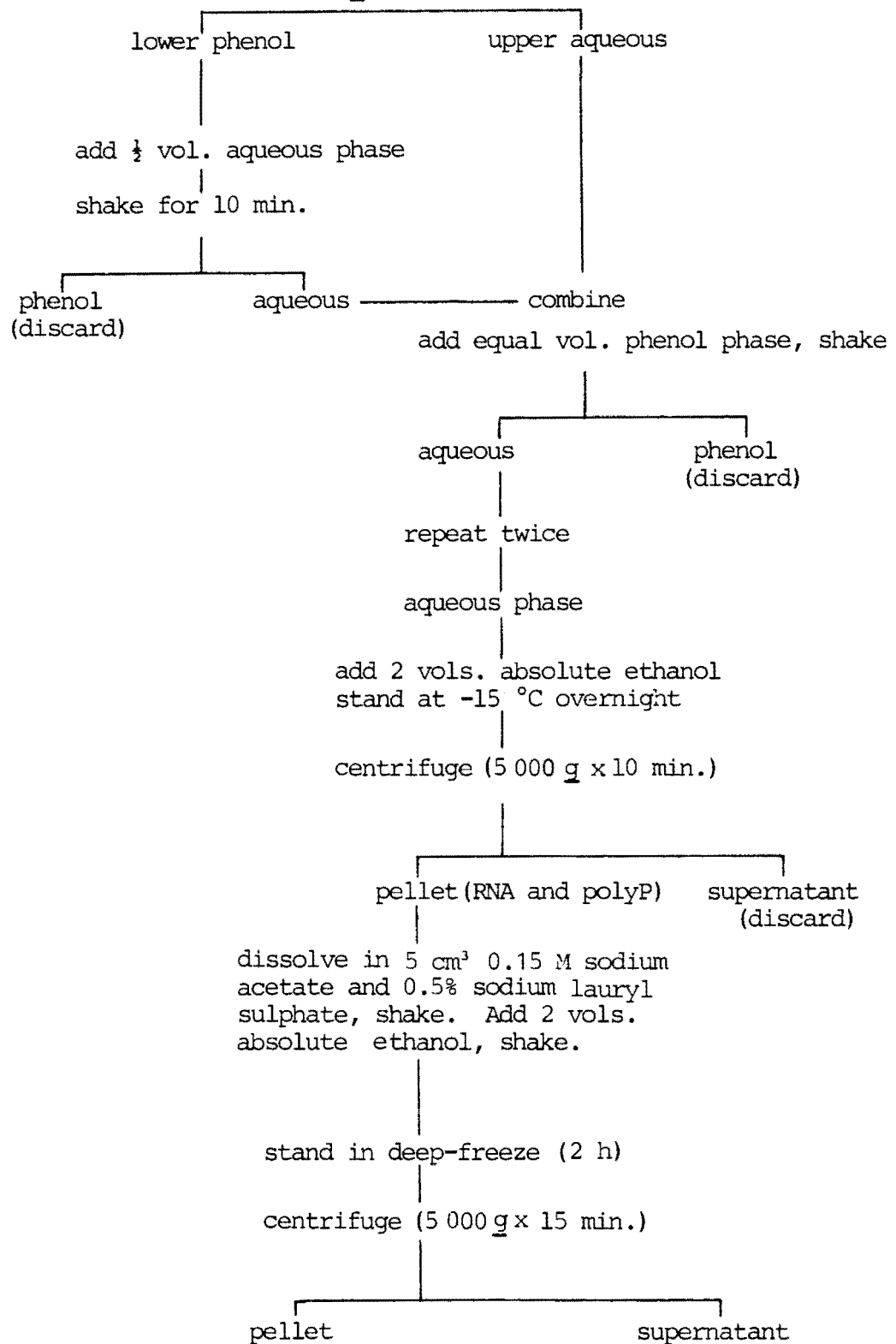
PolyP granules were observed in the hyphae of endophytes by using the staining and extraction techniques of Ashford et al. (1975) and Ling-Lee et al. (1975). Hyphae were teased from mycelial mats, stained for 5 min in a solution of 0.05% toluidine blue adjusted to pH 1.0 with 10 M HCl, rinsed briefly in 0.1 M HCl and mounted in glycerine. Hyphae were also incubated for 15 min in 20% lead nitrate (pH 3.4), rinsed thoroughly in water for 15 min and then stained in 10% ammonium sulphide for 5 min.

### 2.4.2 Phenol-detergent extraction of undegraded nucleic acid-polyP co-precipitates

Extracts of mycelial cultures and seedlings were obtained by the method described by Callow et al. (1978) and summarised in Fig.2.8. The final pellet contained polyP and ribonucleic acid and was dissolved in 0.5 <sup>3</sup>cm 10% sucrose in 0.01 M Tris-HCl buffer (pH 7.8).

Grind 1 g fresh material in 10 cm<sup>3</sup> mixed aqueous  
and phenol phases (5 cm<sup>3</sup> + 5 cm<sup>3</sup>)

Centrifuge 10 000 g x 15 min.)



**FIG. 2.8** Summary of phenol-detergent extraction procedure for  
nucleic acid-polyP co-precipitates

#### 2.4.3 Polyacrylamide gel electrophoresis

Polyphosphates and nucleic acids were separated by gel electrophoresis. The method was similar to that used by Callow et al. (1978) except that electrophoresis was performed on 8.5% (w/v) acrylamide gels using a vertical flat-bed apparatus similar to that described by Reid and Bielecki (1968). After pre-electrophoresing for 2 h at 10 mA constant current, 30  $\mu$ l samples were loaded on to the gel and run for 15 min at 15 mA followed by up to 2 h at 30 mA. Gels were run at 10 °C, then stained by immersion in 0.1% toluidine blue in 1% acetic acid. After staining and destaining, individual pink (polyP) and blue (nucleic acid) bands were scanned between 500 and 700 nm in a Pye Unicam SP1800 spectrophotometer. Gels were scanned using a Vitatron densitometer at fixed wavelengths closest to absorption maxima. An extract (30  $\mu$ l) was run on an 8.5% gel with a range of synthetic sodium polyP compounds (sodium phosphate glasses,  $\text{Na}_{n+2} \text{P}_n \text{O}_{3n+1}$  Types 35, 45, 65, 135; Sigma Chemical Co.) of known molecular weights. The logarithmic relationship between the distance of migration (determined from densitometer scans) and molecular weight of the markers was expressed in the form of a power curve equation.

#### 2.4.4 Total P and acid-labile polyP determinations

Mycelial cultures, seedlings and pink-staining gel segments were digested with a tri-acid mixture (10 parts  $\text{HNO}_3$  : 1

part  $\text{H}_2\text{SO}_4$  : 4 parts  $\text{HClO}_3$ ) at 150 to 180 °C. Orthophosphate was assayed by the colorimetric method of Kempers (1975) (Fig. 2.6).

Ribonucleic acid (RNA) in samples of the extracts were adsorbed onto activated charcoal by the method of Bennett and Scott (1971) before the hydrolysis of the acid-labile polyP in 1 M HCl at 100 °C for 10 min. Orthophosphate was then assayed by the method of Kempers (1975).

#### 2.4.5 Statistical analysis

Size classes of metachromatic granules were obtained from three separate cultures, each culture representing 40 to 80 random measurements from younger, marginal hyphae. Percentages were converted to arcsin transformations. The original measurements were subjected to a computer-based programme of Correspondence Analysis (Greenacre, 1984). All other granule values were obtained from 3 separate cultures, each culture representing 10 to 20 random counts. Values given for the phenol-detergent extraction of the E. hispidula endophyte represent three separate extractions; all other values represent at least three replicates from a single extraction. Molecular weights of polyP molecules were determined from four separate gels for each of the three endophytes used.

#### 2.4.6 Phosphorus extraction and fractionation procedures

A modified version of the Aitchison and Butt (1973) fractionation procedure (Fig. 2.9) was used in experiments which involved the extraction and fractionation of P.

#### 2.4.7 $^{32}\text{P}$ absorption and utilization by mycelia in culture

Fresh mycelia were washed in ice-cold, distilled water, blotted dry and weighed immediately. When the incubation medium did not contain fresh basal medium, mycelia were incubated in 3 cm<sup>3</sup> of  $5 \times 10^{-4}$  M CaSO<sub>4</sub> at room temperature for 15 min before transfer to the radioactive incubation medium. Incubation took place in a shaking water-bath at 25 °C and mycelia were washed in 10 cm<sup>3</sup> distilled water, blotted dry and fractionated. The procedures for solubilization of non-aqueous samples, liquid scintillation counting and the analysis of data are described in section 2.3.1 of this chapter. Final results are expressed as nmol  $^{32}\text{P}$  g<sup>-1</sup> fresh mass.

#### 2.4.8 Total P determination of non-radioactive fractions

In the experiment to assess the endogenous P status of fractions of fresh mycelia, total P of the fractions was determined by the method of Kempers (1975) after digestion (section 2.4.4).

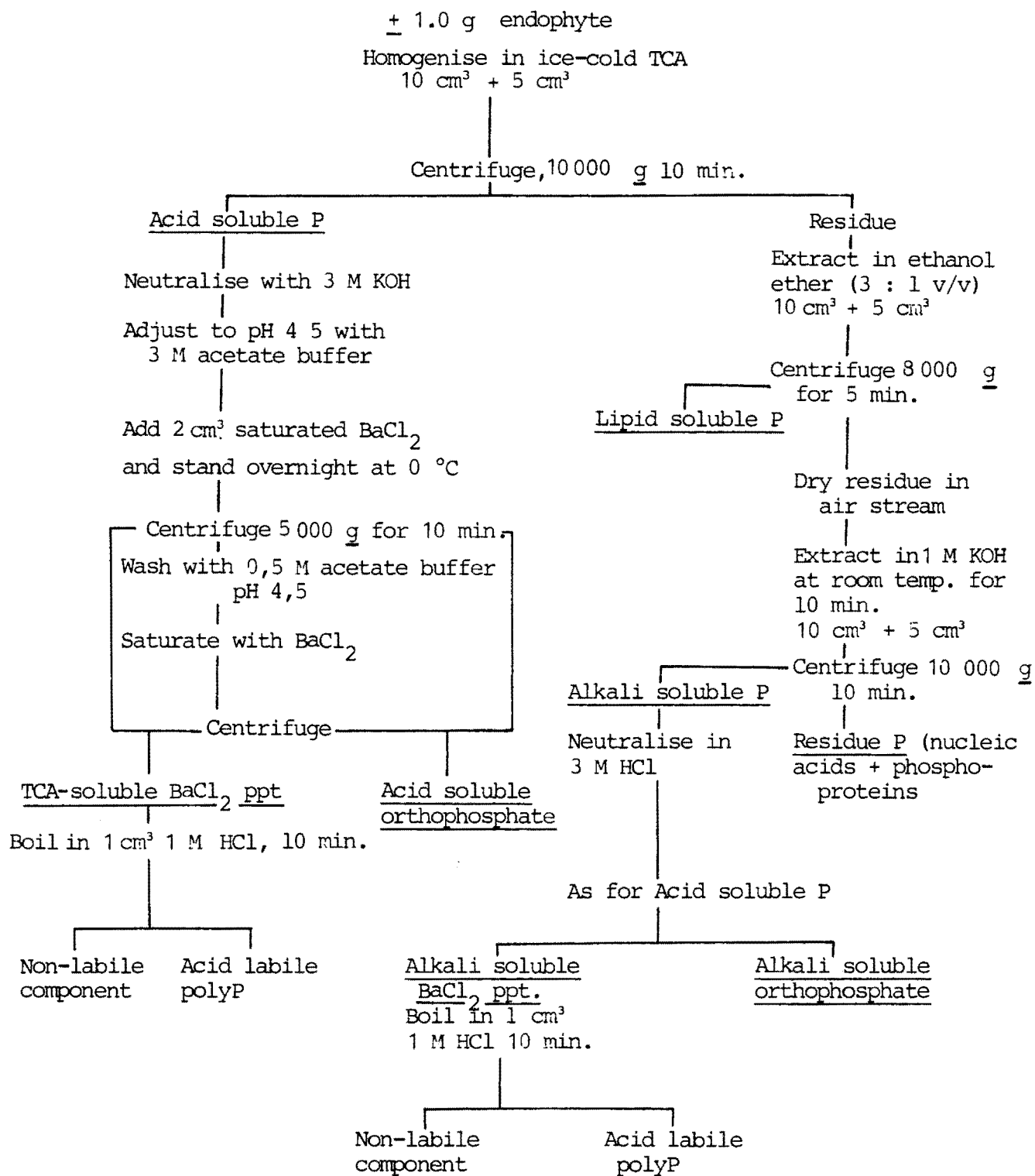


FIG. 2.9 Summary of phosphorus extraction and fractionation procedure based on that of Aitchison and Butt (1973)

#### 2.4.9 The effect of activated charcoal on the adsorption of P contaminants of polyP fractions

To assess the potential for contamination of polyP fractions by unknown P compounds, cultures of the same batch were separated into two groups and fractionated. In one set of extracts, suspensions of the TCA-soluble and -insoluble supernatants containing activated charcoal (0.3g per 5 cm<sup>3</sup>) were covered with 0.01 cm<sup>3</sup> of 10% Triton X-100, centrifuged and filtered (Crane, 1958). Final P levels of the fractions were then compared with those of the set of untreated extracts.

#### 2.4.10 Phytic acid determination

The level of phytic acid in mycelia from the same batch of cultures used in part 2.4.9 was determined by the method of Allen et al. (1974). An acidified extract dissolved any phytin present and phytic acid was isolated as ferric phytate which was recovered, digested and estimated as phytate-phosphorus (see section 2.4.4).



## CHAPTER 3

### ACID PHOSPHATASE ACTIVITY IN ISOLATED ERICOID ENDOPHYTES

#### 3.1 INTRODUCTION

Acid phosphatases are universally found in plants and microorganisms and their activity in the soil may be correlated with high amounts of organic carbon and organic phosphorus and low levels of free phosphate ions (Spiers and McGill, 1979; Appiah and Thomas, 1982). These enzymes of wide specificity, facilitate the hydrolytic cleavage of phosphate-ester bonds and have been accorded an important role in the mineralization of the organic P fraction in soils. Mycorrhizal roots which possess a high acid phosphatase activity may be more efficient in the utilization of soil phosphorus than non-mycorrhizal roots. High acid phosphatase activity has been measured in ectomycorrhizal fungi in pure culture (Theodorou, 1971; Ho and Zak, 1979; Calleja et al., 1980) excised ectomycorrhizal roots (Woolhouse, 1969; Bartlett and Lewis, 1973) and onion roots infected with vesicular-arbuscular mycorrhizas (Gianinazzi et al., 1979). The only report of acid phosphatase activity in ericoid mycorrhizas is that of Pearson and Read (1975) who found high levels of the enzyme in the endophyte of C. vulgaris. This chapter investigates acid phosphatases in mycelia of South African and European endophytes. The high activity of an acid phosphatase

secreted into the external medium of cultures of the South African endophyte prompted a more detailed characterization of the enzyme in cytoplasmic, wall- and membrane-bound and extracellular fractions.

### 3.2 RESULTS

#### 3.2.1 Growth curves of the South African endophytes

The growth curves of European endophytes on different P sources have been determined (Mitchell and Read, 1981) and it was necessary to initially establish if the two South African endophytes have similar growth characteristics. The endophytes isolated from E. hispidula and E. mauritanica grew exponentially in liquid culture up to 14 days after inoculation and then entered the stationary phase (Fig. 3.1). Both endophytes yielded a greater dry mass on orthophosphate than on sodium phytate. The slowest growth was obtained by the E. mauritanica endophyte on a basal medium containing sodium phytate. By day 14, the pH of the growth media containing organic or inorganic P had dropped to between 2.5 and 3.0 for both endophytes.

#### 3.2.2 Acid phosphatase activity and protein content of mycelial fractions of European and South African endophytes

The mycelia of European and South African endophytes were fractionated into wall- and membrane-bound, cytoplasmic and extracellular phases. Protein levels of the fractions of

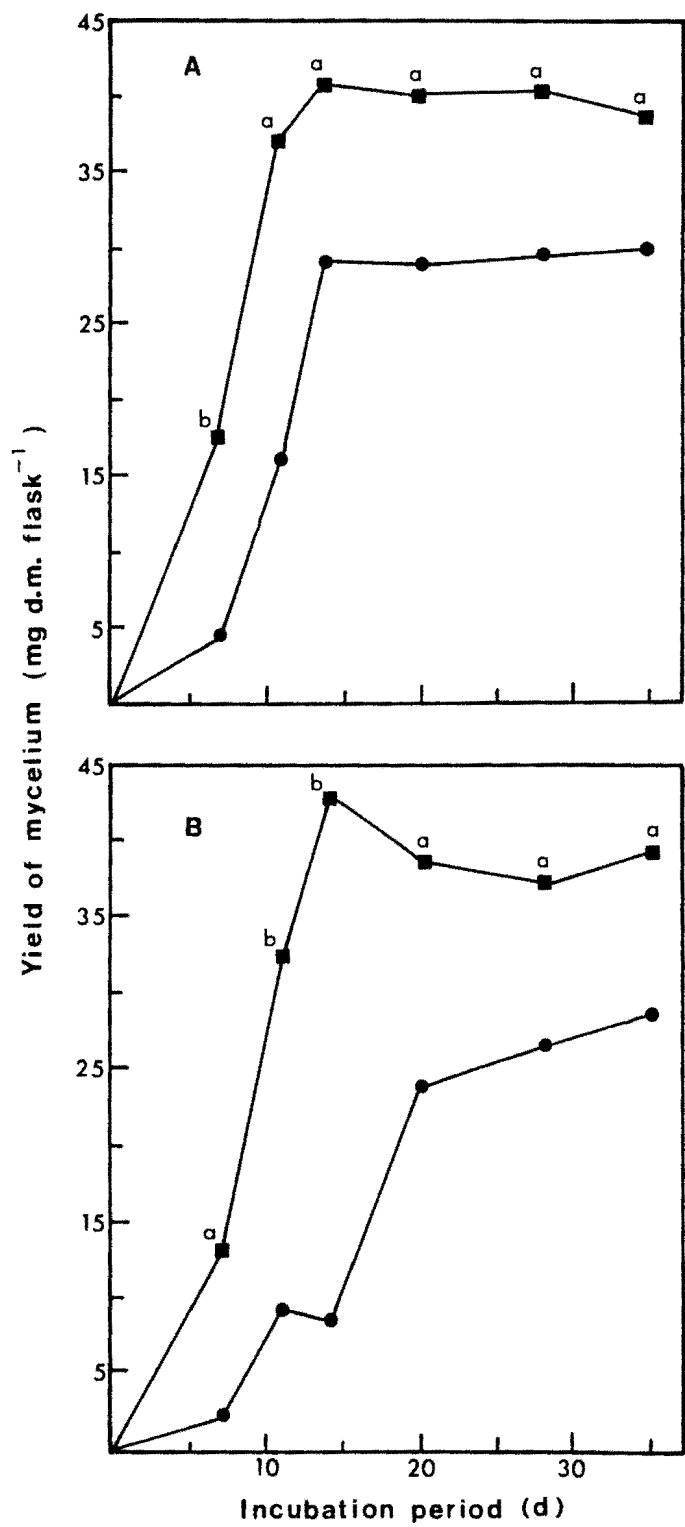


FIG. 3.1 Growth curves of mycelia of the endophytes of (A) *Erica hispidula* and (B) *Erica mauritanica* grown on two different forms of P: orthophosphate (■—■) and sodium phytate (●—●). The symbols a and b denote significance levels between growth on the two forms of P at  $p \leq 0.01$  and  $p \leq 0.05$  respectively. Results are the means of 3 replicates.

all four endophytes showed an initial lag phase of seven days with maximum levels being attained 15 to 20 days after inoculation (Fig. 3.2). Wall- and membrane-bound protein was considerably higher than extracellular and cytoplasmic protein in the four endophytes during all the growth phases. In cultures of the endophytes of C. vulgaris, R. ponticum and V. macrocarpon, there were few differences between cytoplasmic and extracellular protein levels whereas cytoplasmic protein was very low in the endophyte of E. hispidula.

Acid phosphatase activity of extracellular, cytoplasmic and wall- and membrane-bound fractions of the four endophytes are shown in Fig. 3.3. In the endophytes of C. vulgaris, R. ponticum and V. macrocarpon, wall- and membrane-bound phosphatase was the most active enzyme after an initial lag phase of seven days and maximum activity was attained fourteen days after inoculation. Wall- and membrane-bound phosphatase activity in the endophyte of V. macrocarpon was maintained during the final phases of growth despite the fact that total protein content declined (Figs. 3.2 and 3.3). The presence of extracellular phosphatase was demonstrated in all cultures seven days after inoculation although the activity of this enzyme was highest in the endophyte of E. hispidula (Fig. 3.3). At maximum activity, the ratios of extracellular, cytoplasmic wall- and membrane-bound phosphatase of the endophyte of E. hispidula to those

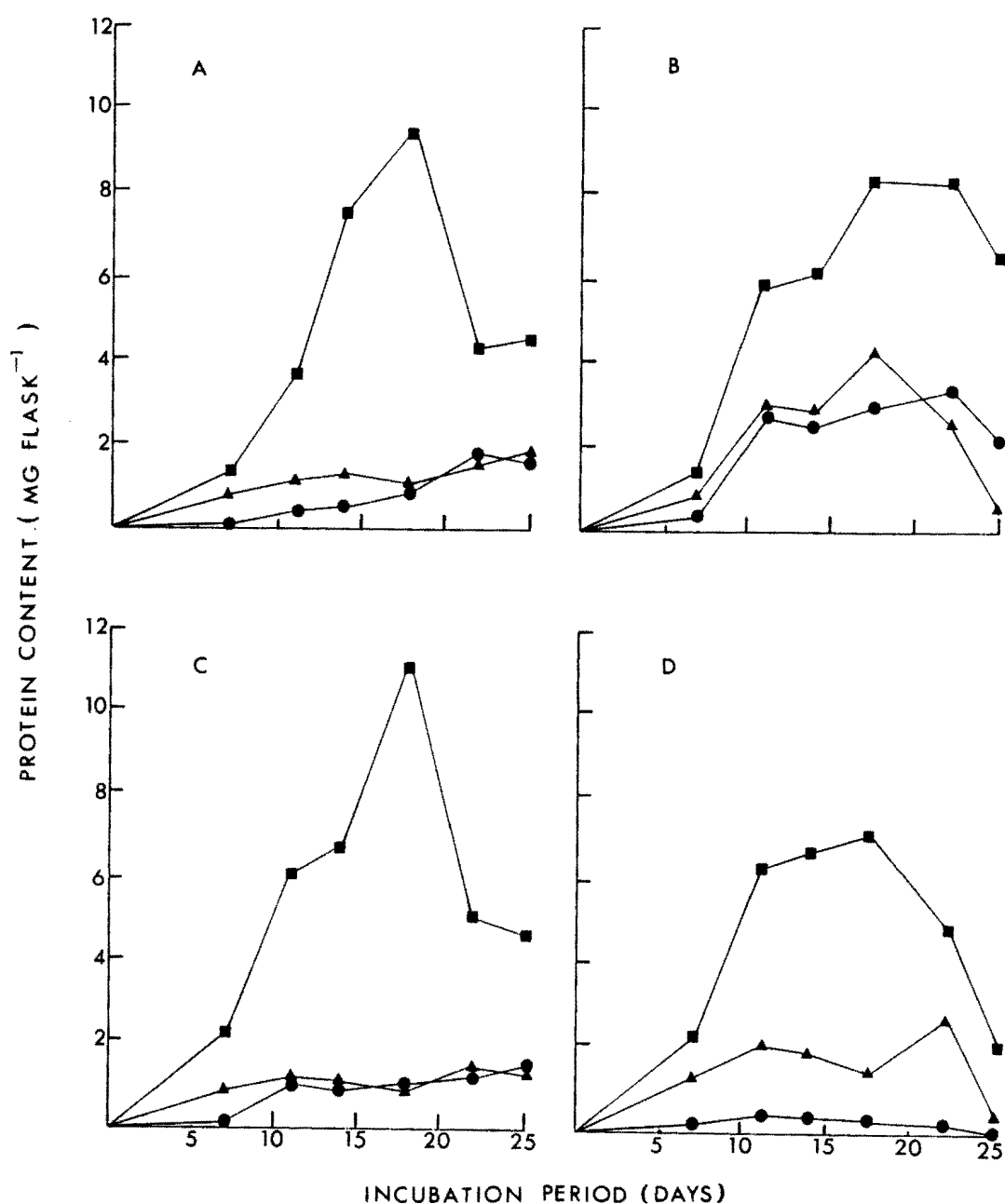
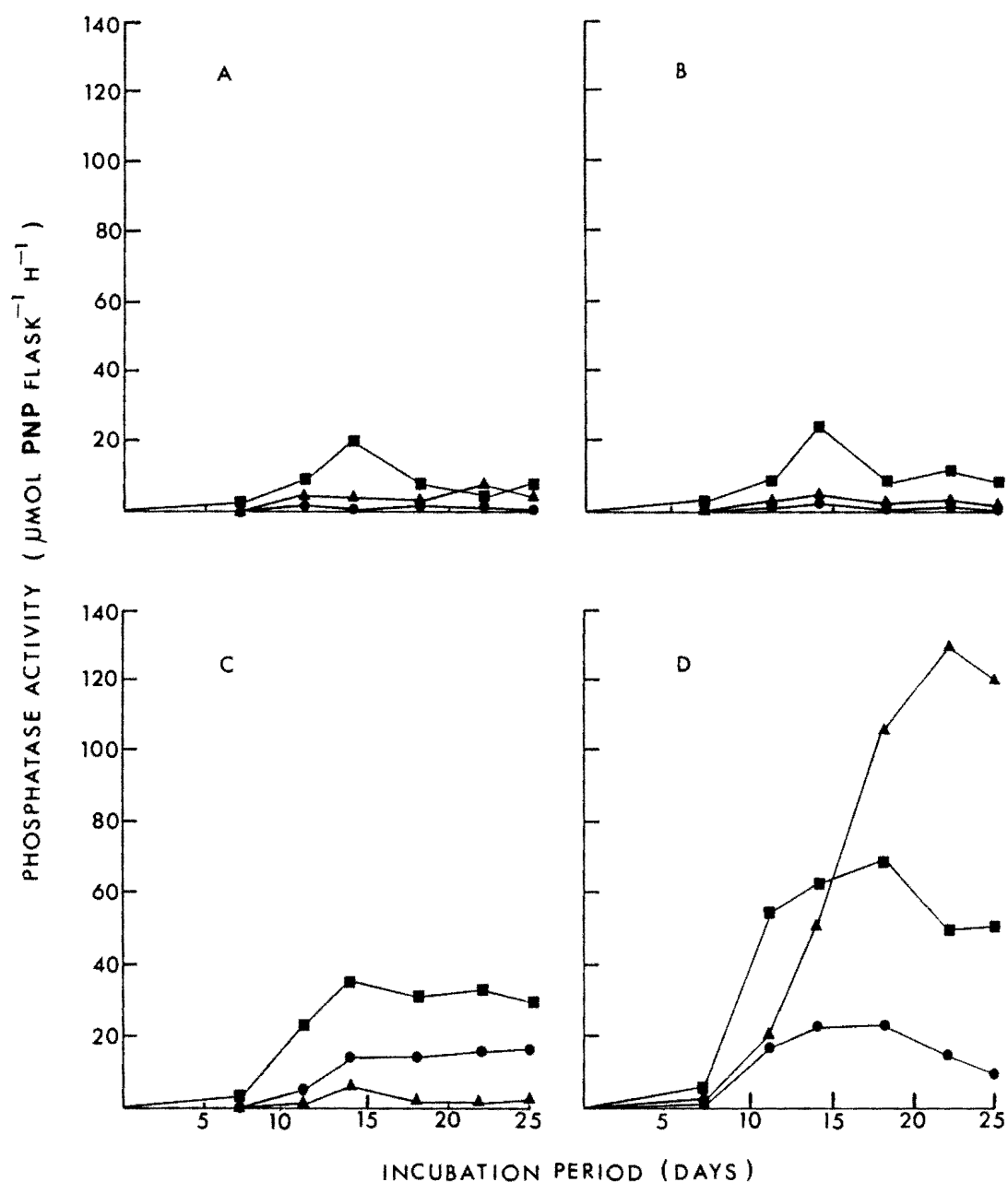


FIG. 3.2 Protein levels of wall- and membrane-bound (■—■), cytoplasmic (●—●), and extracellular (▲—▲) fractions of endophytes: A, *Rhododendron ponticum*; B, *Calluna vulgaris*; C, *Vaccinium macrocarpon* and D, *Erica hsipidula*. Results are the means of 5 replicates (each replicate representing fractions from different cultures).



**Fig. 3.3** Acid phosphatase activity of wall- and membrane-bound (■—■), cytoplasmic (●—●) and extracellular (▲—▲) fractions of endophytes: A, *Rhododendron ponticum*; B, *Calluna vulgaris*; C, *Vaccinium macrocarpon* and D, *Erica hispidula*. Results are the means of 5 replicates (each replicate representing fractions from different cultures).

of V. macrocarpon were 20.5, 1.6 and 2 respectively. Extracellular phosphatase in the endophyte of E. hispidula was more active than the cytoplasmic and wall- and membrane-bound enzymes 18 days after inoculation.

### 3.2.3 Phosphatase activity in fractions of mycelia of the endophyte of Erica hispidula

In the previous section the endophyte of E. hispidula had the highest total acid phosphatase activity when compared with three European endophytes and was thus selected for further studies. To determine the influence of growth under different organic P conditions on phosphatase activity, fractions were extracted from both high P (3.23 mM Na phytate)- and low P (0.1 mM Na phytate)-fed mycelia. The extraction procedure of Hasegawa et al. (1976) was adopted to achieve separation of wall-bound enzymes from the wall debris (see section 2.1.2). It was found that the ability of NaCl to solubilize wall-bound proteins was dependent on concentration (Fig. 3.4) with the highest degree of solubilization occurring at 1 M NaCl.

A comparison of the acid phosphatase activity of the crude extracts expressed on a fresh mass or total protein basis and measured immediately after extraction is given in Table 3.1. In high P-fed mycelia the activity of the extracellular fraction was 5.5 times greater than that of the total wall and 19 and 13 times greater than that of the cytoplasmic and

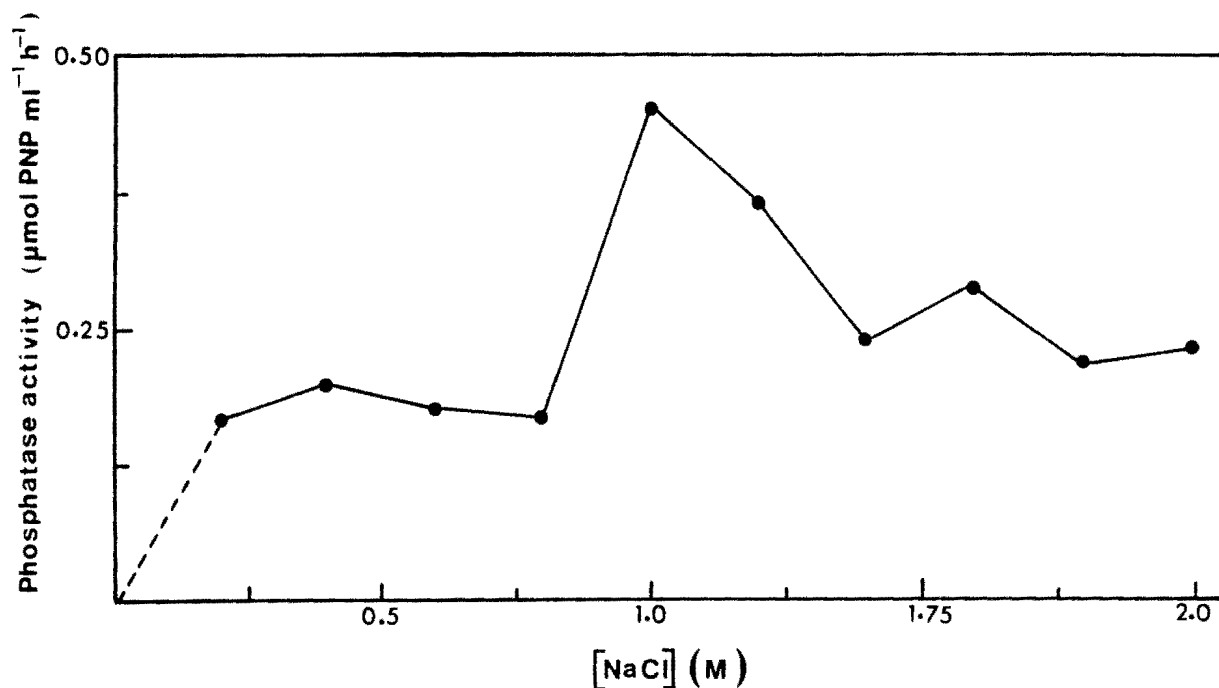


FIG. 3.4 Effect of NaCl on the quantity of acid phosphatase released from the cell wall debris of mycelia of the endophyte of *Erica hispidula*. Aliquots of debris suspensions were incubated in NaCl overnight at 0°C, centrifuged at 12 000 g for 10 min and the supernatant assayed for phosphatase activity. Values presented are averages of duplicate experiments.



TABLE 3.1 The acid phosphatase activity in fractions of the mycelia of the endophyte of *Erica hispidula* at different stages of purification

Fraction	Activity <sup>a</sup> ( $\mu\text{mol PNP g}^{-1}$ fresh mass $\text{min}^{-1}$ ) $\pm$ SE ( $\text{nmol PNP mg}^{-1}$ protein $\text{min}^{-1}$ ) $\pm$ SE	
	High P mycelia	Low P mycelia
<u>Extracellular</u>		
Crude extract	5987 $\pm$ 330 (76) <sup>b</sup> 497 $\pm$ 30 (75)	2000 $\pm$ 40 (36) 135 $\pm$ 3 (36)
Dialysis + lyophilisation	321 $\pm$ 70 27 $\pm$ 6.4	849 $\pm$ 20 57 $\pm$ 1
Sephacryl S-400	55 $\pm$ 0.8 4.3 $\pm$ 0.3	150 $\pm$ 3 8 $\pm$ 0.2
<u>Cytoplasmic</u>		
Crude extract	455 $\pm$ 10 (6) 39 $\pm$ 1 (6)	427 $\pm$ 30 (8) 28 $\pm$ 2 (8)
Dialysis + lyophilisation	507 $\pm$ 50 43 $\pm$ 4	22 $\pm$ 1 1.4 $\pm$ 0.07
Sephacryl S-400	115 $\pm$ 2 10 $\pm$ 0.5	0 0
<u>Membrane</u>		
Crude extract	334 $\pm$ 10 (4) 28 $\pm$ 1 (5)	590 $\pm$ 6 (11) 43 $\pm$ 1 (11)
Dialysis + lyophilisation	51 $\pm$ 4 43 $\pm$ 0.4	22 $\pm$ 0 1.5 $\pm$ 0.02
<u>Wall (NaCl-soluble)</u>		
Crude extract	1029 $\pm$ 30 (13) 83 $\pm$ 5 (13)	1941 $\pm$ 50 (35) 131 $\pm$ 4 (35)
Dialysis + lyophilisation	709 $\pm$ 100 60 $\pm$ 8	334 $\pm$ 20 23 $\pm$ 1.4
Sephacryl S-400	269 $\pm$ 5 18 $\pm$ 0.4	89 $\pm$ 4 (peak I) 6 $\pm$ 0.2 13 $\pm$ 0.7 (peak II) 0.9 $\pm$ 0.07
<u>Wall (NaCl-insoluble)</u>		
Crude extract	83 $\pm$ 10 (1) 4.4 $\pm$ 0.8 (1)	581 $\pm$ 29 (10) 39 $\pm$ 2 (10)
<u>Total</u>	7888 (100) 627 (100)	5539 (100) 376 (100)

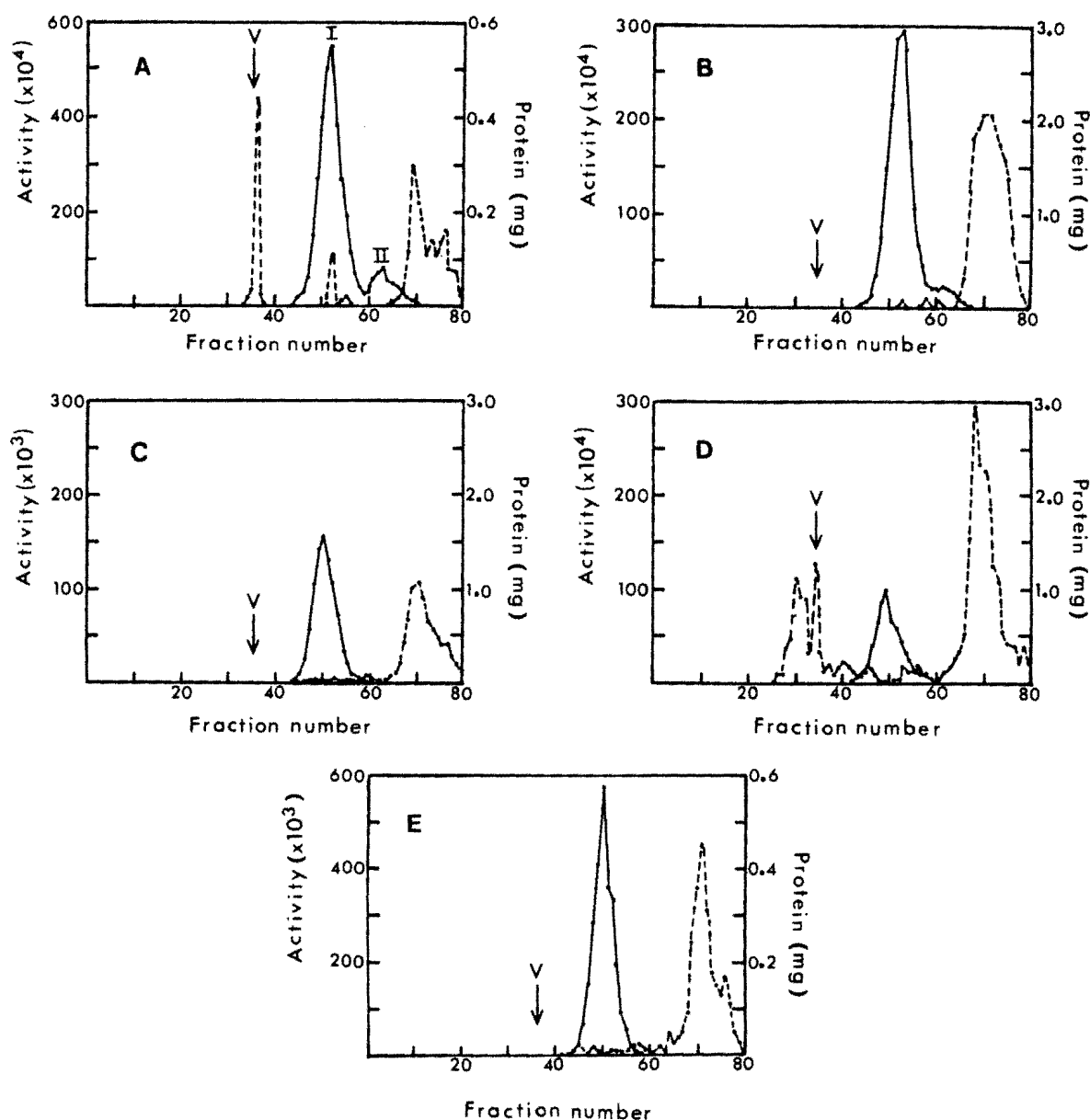
<sup>a</sup>Results are the means of 3 or 4 replicates

<sup>b</sup>Figures in parentheses represent percentage contribution of each fraction to total activity

membrane-bound fractions respectively. In high P-fed mycelia, the activity of the extracellular fraction was 77% of total activity. The activity of the extracellular and soluble wall fractions of low P-fed mycelia were similar and together formed 80% of total activity. The low P extracellular fraction was 4.5 and 3.3 times more active than the low P cytoplasmic and membrane fractions respectively. It appears that 92% of the wall-bound phosphatase enzymes of high P-fed mycelia were solubilized by 1 M NaCl whereas for low P-fed mycelia it was 77%.

#### 3.2.4 Gel filtration

Samples of partially purified extracts (section 2.1.2) were eluted from a Sephracryl S-400 gel column to further characterise the enzymes of the different fractions. Single peaks eluted from all extracts except the low P soluble wall fraction which produced a major peak coinciding with the single peak of the other fractions and a minor one of a smaller molecular weight (Fig. 3.5). The acid phosphatase activity of the membrane-bound and the low P cytoplasmic fractions could not be detected after elution through the column even after further concentration by lyophilisation. Molecular weight values corresponding to the elution peaks were estimated by eluting four markers (catalase, ovalbumen, bovine albumen and cytochrome C) through the column (Fig. 3.6). The dominant peak, obtained from all the extracts corresponded to a molecular weight of  $173\,858 \pm 8592$  (high



**FIG. 3.5** The elution profiles of protein (-----) and acid phosphatase activity (—) following gel filtration of fractions of cultures of the endophyte of *Erica hispidula* on a Sephacryl S-400 column: (A) low P, wall; (B) low P, extracellular; (C) high P, wall; (D) high P, extracellular; (E) high P, cytoplasmic. Activity is expressed in  $\mu\text{mol PNP mg}^{-1} \text{ protein min}^{-1}$ . Protein was assayed by the method of Lowry et al. (1951). The symbol V denotes the void volume of the column determined with Blue Dextran 2000. Protein is  $\text{mg } 3 \text{ cm}^{-3}$  aliquot eluted.

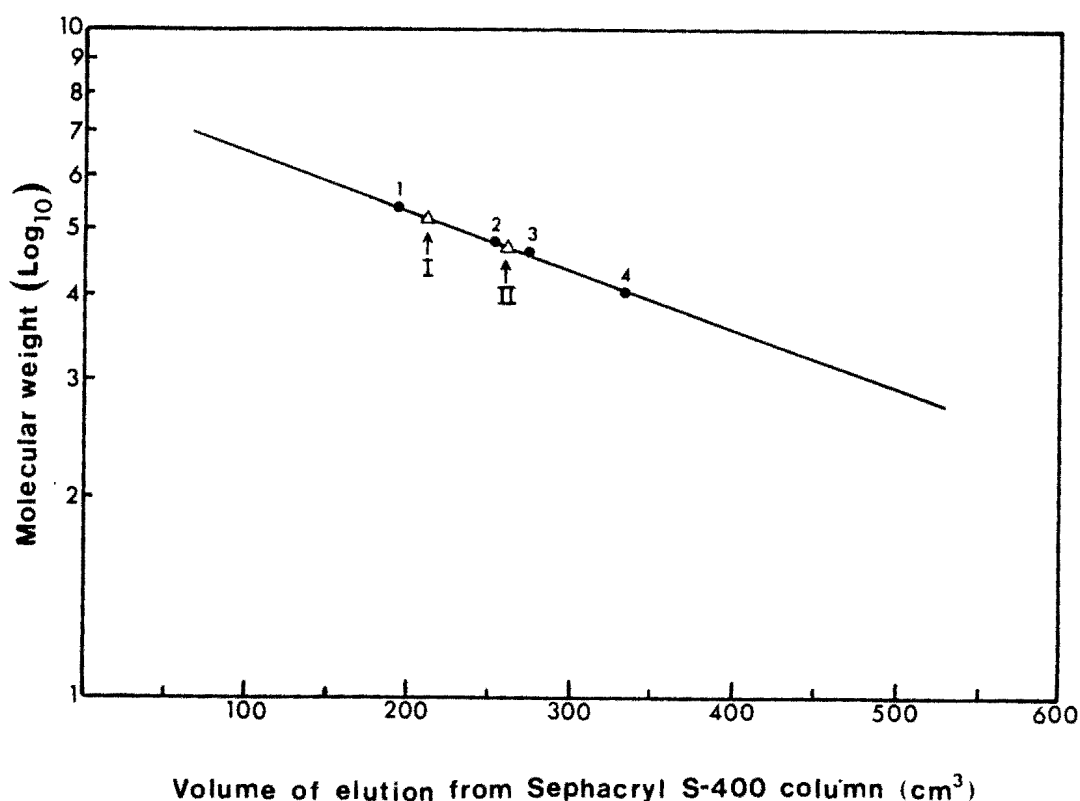


FIG. 3.6 Molecular weight estimation of acid phosphatase peaks eluted from Sephacryl S-400 column. Molecular weight I (dominant peak, representative of all fractions) and II (subsidiary peak, present only in low P mycelia) was estimated from a standard curve ( $y = 0.01x + 7.17$ ,  $r = 0.91$ ) prepared using the following standards: (1) catalase (232 000); (2) bovine albumin (66 200); (3) ovalbumin (45 000); (4) cytochrome C (13 096). Volumes of standards were the means of duplicate elutions and molecular weight estimates are the means of at least 3 replicates. Protein was assayed by the method of Lowry *et al.* (1951). The technique of molecular weight estimation is that used by Basha (1984).

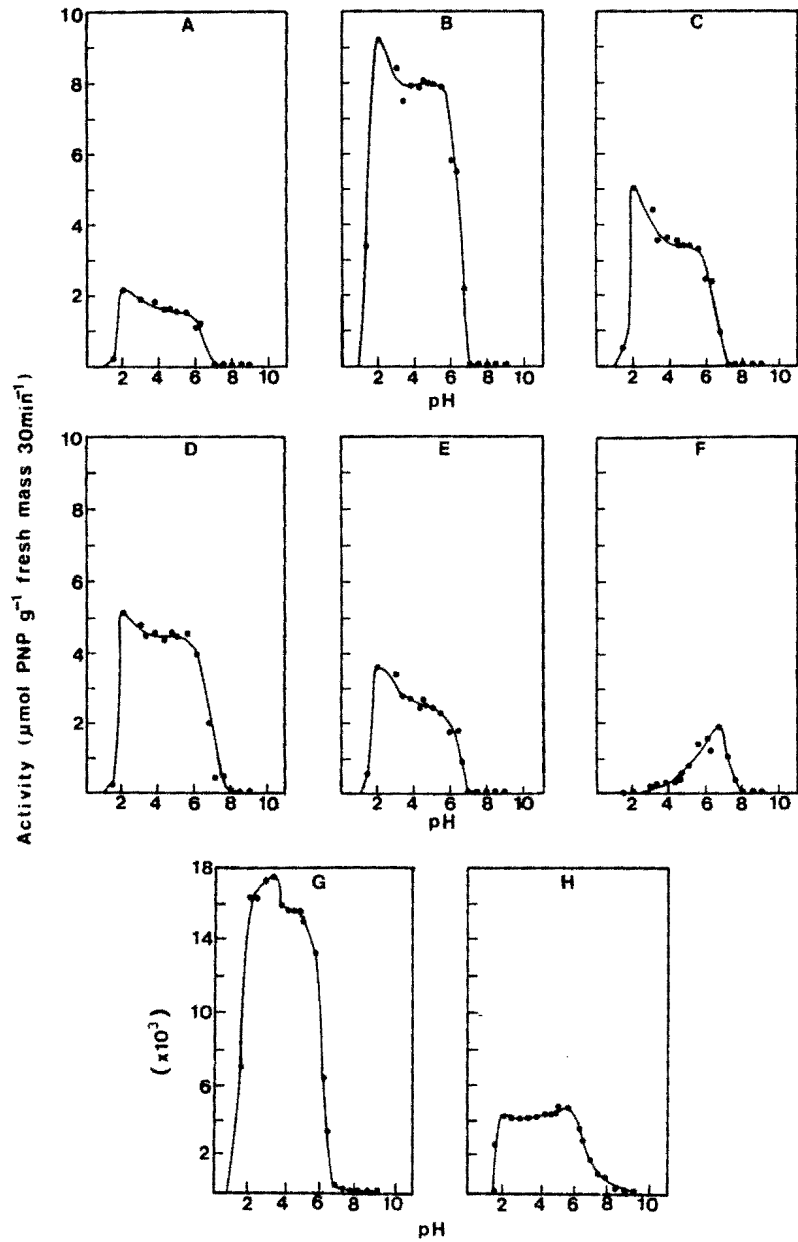
molecular weight enzymes) whereas the minor peak only eluted from the low P soluble wall extract had a molecular weight of  $68\,028 \pm 3348$  (low molecular weight enzyme).

### 3.2.5 pH scans

The buffer routinely used for the assay of acid phosphatase was pH 4.5 (Pearson and Read, 1975) but this might not have been the optimum pH for all the enzymes extracted from mycelia of the endophyte of E. hispidula. Moreover, pH serves as a basis for distinguishing the properties of the extracellular, soluble wall and cytoplasmic fractions. All the fractions, except the low P soluble wall II enzyme hydrolysed PNPP optimally between pH 2.0 and 6.0 with a peak in activity at pH 2.0 (Fig. 3.7). The activity of these enzymes declined rapidly above pH 5.5 to negligible rates at pH 7.0. The low P, soluble wall II enzyme showed an optimum activity at pH 6.5 with a rapid decline at pH 8.0. (Fig. 3.7)

### 3.2.6 The action of effectors

The action of different compounds as either possible activators or inhibitors of the acid phosphatases are shown in Table 3.2. Although variations between enzymes in the degree of response to different agents was observed, the clearest distinction is between the low molecular weight enzyme and the high molecular weight enzymes as a group. The agents which stimulated the activity of the high molecular weight enzymes most were ferric ions in low



**FIG. 3.7** pH scans of phosphatase activity in fractions of mycelia of the endophyte of *Erica hispidula*: (A) high P, extracellular; (B) high P, soluble wall; (C) high P, cytoplasmic; (D) low P, extracellular; (E) low P, soluble wall (peak I); (F) low P, soluble wall (peak II); (G) high P, membrane-bound; (H) low P, membrane-bound. A-F scanned after elution through a Sephacryl S-400 column; G and H were not eluted through the gel filtration column. Data points are the means of 4 replicates.

**TABLE 3.2** The influence of various reagents on the acid phosphatase activity of fractions of the endophyte of *Erica hispidula* grown for 12 d on basal medium and either 3.23 mM (high P) or 0.1 mM (low P) sodium phytate

Reagent	Conc. (mM)	Relative activity (%) <sup>a</sup> of fractions					
		Cytoplasmic High P	Extracellular High P	Low P	Wall (soluble) High P Low PI Low PII		
EDTA	5.0	121	164	128	127	122	93
	25.0	133	168	142	145	129	80
Potassium citrate	5.0	144	161	143	155	118	99
	25.0	150	162	143	149	126	77
Magnesium chloride	1.0	97	99	95	104	105	67
	25.0	84	98	141	99	102	84
Calcium chloride	1.0	97	102	107	85	101	48
	25.0	98	104	107	99	105	93
Ferric chloride	0.5	121	115	111	108	104	111
	10.0	48	53	47	80	41	0
Copper sulphate	0.1	87	97	84	92	89	112
	25.0	45	59	50	51	51	10
Zinc sulphate	1.0	82	77	79	103	86	118
	25.0	72	77	67	99	79	85
Nickel sulphate	5.0	103	88	104	59	94	59
	25.0	99	87	104	53	96	65
Cobalt sulphate	5.0	39	30	39	45	39	108
	10.0	59	55	68	70	57	67
Mercuric chloride	1.0	72	69	64	92	73	-
	25.0	16	23	18	25	26	-
Sodium arsenate	5.0	53	49	51	55	46	0
	25.0	11	9	9	11	9	0
Sodium cyanide	5.0	84	70	85	93	82	0
	10.0	11	7	36	14	25	0
Sodium fluoride	5.0	0	0	0	0	0	48
Sodium molybdate	5.0	0	0	0	0	0	0
Sodium phosphate	5.0	59	58	58	69	58	20
	25.0	25	25	24	27	25	3
Sodium nitrate	5.0	93	91	84	92	91	113
	25.0	94	102	98	105	102	98

<sup>a</sup> Activity expressed as a percentage of the control. Activity assessed under standard conditions using PNPP as substrate. Reagents were dissolved in 0.1 M maleate buffer (pH 6.5) for assay of low P, wall-bound II enzyme or 0.1 M glycine/HCl buffer (pH 2.2) for assay of other enzymes. Low PI and low PII refer to the high and low molecular weight peaks respectively.

concentrations, EDTA and citrate whereas ferric and cupric ions in low concentrations stimulated the activity of the low molecular weight enzyme. Fluoride and molybdenum inhibited all the enzymes whereas the degree of inhibition by phosphate, cyanide, arsenate, mercury, cobalt, copper and ferric ions was dependent on concentration. Magnesium, calcium and nickel ions caused little variation in the activity of the high molecular weight enzymes but in low concentrations inhibited the activity of the low molecular weight enzyme.

### 3.2.7 Substrate specificity

Acid phosphatases generally show affinities for a broad range of substrates but may differ substantially in their affinities for specific substrates. The activities of six acid phosphatase fractions from mycelia of the endophyte, E. hispidula, towards various substrates are shown in Table 3.3. The substrate most efficiently hydrolysed by all the fractions was inorganic pyrophosphate with  $\alpha$ -glycerophosphate,  $\alpha$ - and  $\beta$ -naphthyl phosphate, phenol phosphate, PNPP and  $\beta$ -glycerophosphate also showing high rates of hydrolysis. All the fractions except the low P, soluble wall II fraction showed a high affinity for glucose-1-phosphate but not for glucose-6-phosphate or fructose 6-phosphate which proved successful substrates for the low P, soluble wall II enzyme. Fructose-1,6-bisphosphate and the organic anhydrides, especially ATP, proved suitable



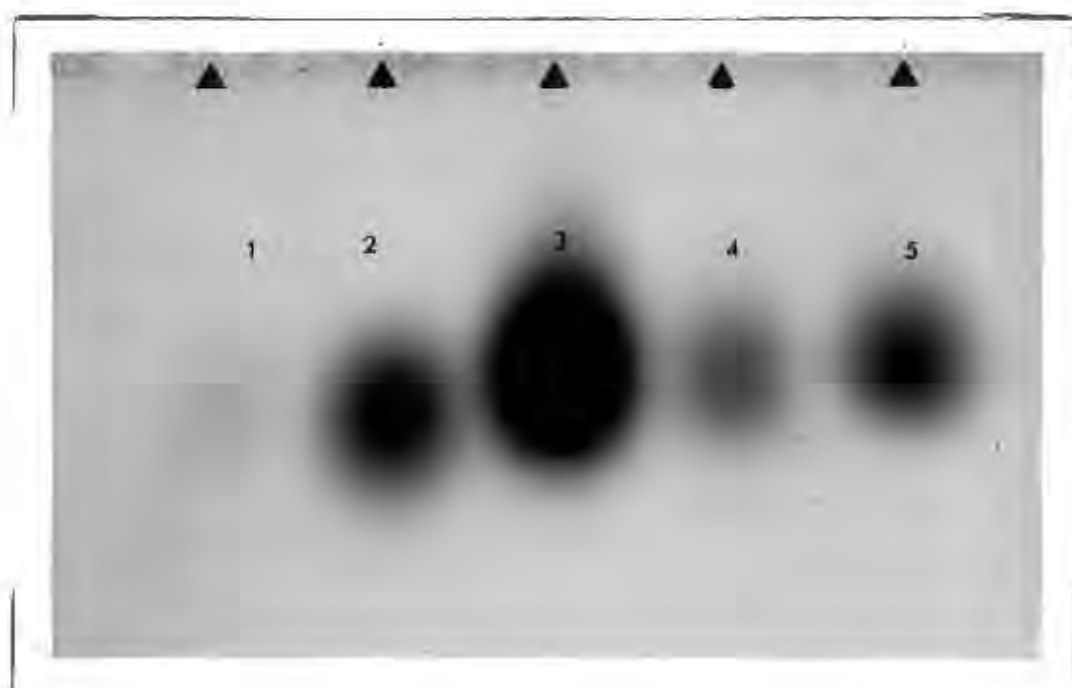
TABLE 3.3 The comparative activities of the acid phosphatases of the endophyte of *Erica hispidula* grown for 12 d on basal medium and either 3.23 mM (high P) or 0.10 mM (low P) sodium phytate towards various substrates. Enzyme fractions are those eluted from a Sephacryl S-400 gel column

Substrate	Activity of fractions ( $\mu\text{mol P released g}^{-1}$ fresh mass $30 \text{ min}^{-1}$ ) <sup>±</sup> SE					
	Cytoplasmic High P	Extracellular High P	Low P	High P	Wall (soluble) Low PI	Low PII
p-Nitrophenol phosphate (PNPP)	2.21 <sup>±</sup> 0.1(100) <sup>a</sup>	0.96 <sup>±</sup> 0.1(100)	1.69 <sup>±</sup> 0.4(100)	4.65 <sup>±</sup> 0.4(100)	1.66 <sup>±</sup> 0.09(100)	1.28 <sup>±</sup> 0.07(100)
DL- $\alpha$ -Glycerophosphate	2.74 <sup>±</sup> 0.2(124)	1.42 <sup>±</sup> 0.2(148)	2.38 <sup>±</sup> 0.2(142)	6.68 <sup>±</sup> 0.7(144)	1.61 <sup>±</sup> 0.2(97)	3.37 <sup>±</sup> 0.4(264)
$\beta$ - Glycerophosphate	1.75 <sup>±</sup> 0.06(79)	0.71 <sup>±</sup> 0.06(74)	1.57 <sup>±</sup> 0.2(93)	4.08 <sup>±</sup> 0.09(88)	0.97 <sup>±</sup> 0.08(59)	0.88 <sup>±</sup> 0.3(69)
Inorganic pyrophosphate	6.12 <sup>±</sup> 0.8(276)	5.62 <sup>±</sup> 2.1(583)	8.82 <sup>±</sup> 0.4(523)	9.93 <sup>±</sup> 0.09(213)	6.00 <sup>±</sup> 0.3(362)	27.10 <sup>±</sup> 1.3(2122)
$\alpha$ - Naphthyl phosphate	3.06 <sup>±</sup> 0(138)	1.90 <sup>±</sup> 0.1(197)	3.25 <sup>±</sup> 0.2(193)	7.05 <sup>±</sup> 0.04(151)	2.57 <sup>±</sup> 0.2(155)	2.48 <sup>±</sup> 0.4(194)
$\beta$ - Naphthyl phosphate	3.68 <sup>±</sup> 0.05(166)	1.72 <sup>±</sup> 0.1(179)	2.90 <sup>±</sup> 0.2(172)	5.94 <sup>±</sup> 0.3(128)	2.24 <sup>±</sup> 0.2(135)	4.66 <sup>±</sup> 1.0(365)
Phenyl phosphate	3.32 <sup>±</sup> 0.2(150)	1.32 <sup>±</sup> 0.4(137)	3.62 <sup>±</sup> 0(215)	6.51 <sup>±</sup> 0.2(140)	2.30 <sup>±</sup> 0.2(139)	4.09 <sup>±</sup> 0.7(320)
Glucose-1-phosphate	2.09 <sup>±</sup> 0.5(94)	1.04 <sup>±</sup> 0.1(108)	2.30 <sup>±</sup> 0.2(136)	4.66 <sup>±</sup> 0.5(100)	1.65 <sup>±</sup> 0.3(99)	0.18 <sup>±</sup> 0.08(14)
Glucose-6-phosphate	0.92 <sup>±</sup> 0.1(42)	0.72 <sup>±</sup> 0.1(74)	1.68 <sup>±</sup> 0.2(99)	1.41 <sup>±</sup> 0(30)	1.15 <sup>±</sup> 0(69)	2.48 <sup>±</sup> 0.5(194)
Fructose-6-phosphate	0.41 <sup>±</sup> 0.2(19)	0.19 <sup>±</sup> 0.1(19)	0.60 <sup>±</sup> 0.2(35)	1.28 <sup>±</sup> 0(28)	0.44 <sup>±</sup> 0.1(26)	3.72 <sup>±</sup> 0.8(29)
Fructose-1,6-bisphosphate	0	0	0	0	0	2.39 <sup>±</sup> 0.6(187)
Adenosine-5 <sup>1</sup> -triphosphate	0	0	0	0	0	5.19 <sup>±</sup> 0.3(406)
Adenosine-5 <sup>1</sup> -diphosphate	0	0	0	0	0	3.98 <sup>±</sup> 0.9(312)
Adenosine-5 <sup>1</sup> -monophosphate	0	0	0	0	0	1.92 <sup>±</sup> 0.1(151)
Sodium inositol hexaphosphoric acid <sup>b</sup>	0.88 <sup>±</sup> 0.3	0.73 <sup>±</sup> 0.2	1.32 <sup>±</sup> 0.4	1.25 <sup>±</sup> 0.1	1.23 <sup>±</sup> 0	2.11 <sup>±</sup> 0.7

<sup>a</sup>Figures in parenthesis represent rate of hydrolysis as a percentage of rate of PNPP hydrolysis which is taken as 100

<sup>b</sup>Enzymes incubated for 24 h in medium supplemented with 25 mM Na EDTA, Activity expressed as nmol P released g<sup>-1</sup> fresh mass 24 h<sup>-1</sup>

Low PI and low PII refer to the high and low molecular weight peaks respectively.



**FIG. 3.8** Electrophoresis of fractions of high P- and low P-fed mycelia of the endophyte of *Erica hispidula*. (1) low P, soluble-wall II; (2) low P, soluble-wall I; (3) high P, cytoplasmic; (4) high P, extracellular; (5) high P, soluble-wall. Gels were stained for acid phosphatase activity. Arrows represent the points of application. Low P soluble-wall II and low P-soluble wall I refer to low and high molecular weight gel filtration peaks respectively.

high P-fed mycelia was 1.5 times greater than that of low P-fed mycelia which can be attributed entirely to the greater activity of the high P extracellular enzyme. In many plants Pi deficiency has been found to invoke an increase in acid phosphatase activity, part of which is due to the appearance of a cell wall-bound enzyme (Bielecki, 1973; Zink and Veliky, 1979). The studies of Calleja et al. (1980) and Calleja and d'Auzac (1983) have confirmed this for isolated ectomycorrhizal fungi. Under conditions of low Pi availability, these organisms showed a large increase (75%) in the total accessible phosphatase activity which was due to an increased contribution by the cell wall and extracellular enzymes. When the acid phosphatases of onion root systems grown with and without the addition of Pi to the soil and infected with VA mycorrhizas were compared with uninfected roots under similar conditions, no significant differences in activity were found (Gianinazzi - Pearson and Gianinazzi, 1976). A surfeit of organic P in the growth medium did not repress the acid phosphatase activity of the endophyte of E. hispidula but stimulated the further synthesis of an already active externally-released enzyme. In Canadian and Ghanaian soils, high soil phosphatase activity has been correlated with high organic P contents in the soil (Appiah and Thomas, 1982).

In this study, the phosphatase enzymes were partially purified and characterised according to molecular weight,

growth when cell lysis would be expected to be minimal. Thus, in the soil environment this enzyme may well give the endophyte access to substrates normally inaccessible to the host roots. The activities of the extracellular enzymes of the European endophytes were low with the wall- and membrane-bound fraction being the dominant one. It has already been shown that the South African and European endophytes also appear to differ in their ability to utilise phytate salts (Mitchell and Read, 1985). Although both European heathland and South African fynbos soils are low in nutrients, the organic matter and total P content of a typical fynbos soil may be as low as 2% of that of an upland heath soil in the United Kingdom (Read and Mitchell, 1983). Thus the secretion of an extracellular phosphatase by the endophytes may be more important in the phosphorus nutrition of the South African ericas than in the European examples of the Ericales.

## CHAPTER 4

### KINETICS OF PHOSPHATE UPTAKE BY THE ISOLATED MYCORRHIZAL ENDOPHYTE OF ERICA HISPIDULA

#### 4.1 INTRODUCTION

Free phosphate ions in the soil and those made available from bound complexes may be taken up by the external hyphae of ericoid mycorrhizas and either used in metabolism or transformed into stored P compounds such as polyphosphates. Ultimately, some of the absorbed P may be transferred from the internal, matrical hyphae to the cortical cells of the host roots. In this way, the external mycorrhizal hyphae would act as a physical extension of the host root by increasing the absorptive surface available for uptake. The endophyte might also facilitate an efficient P absorption through the nature of its uptake system(s). Since P transport across the plasmalemma has been regarded as a carrier-mediated process, the enzyme kinetic equation of Michaelis-Menten can be applied to estimate the kinetic constants of  $K_m$  and  $V_{max}$  (Epstein and Hagen, 1952; Woolhouse, 1975; Beever and Burns, 1980). These constants give valuable information on the efficiency of ion uptake under defined conditions.

This chapter describes investigations into the simple uptake kinetics of an ericoid endophyte as well as giving estimates

of the kinetic constants associated with P uptake. A dual uptake system has been demonstrated in some fungi and mycorrhizas (see Beever and Burns, 1980) and this investigation verifies the presence of a similar system in an isolated ericoid endophyte. Conclusions are drawn on the adaptive advantages of such a system.

## 4.2 RESULTS

### 4.2.1 Uptake of $^{32}\text{P}$ by mycelia grown on different concentrations of P

The endophyte of E. hispidula was grown in a basal liquid medium containing a range of inorganic phosphate concentrations ( $\text{NaH}_2\text{PO}_4$  : 0-3.23 mM). During the exponential phase of growth, the mycelia were transferred to an incubating medium containing  $\text{KH}_2^{32}\text{PO}_4$  ( $20 \mu\text{Ci dm}^{-3}$ ) for 15 min. Uptake of  $^{32}\text{P}$  demonstrated a curvilinear response with a transition occurring at 0.1 mM P in the original growth medium (Fig. 4.1).

### 4.2.2 The rate of uptake of $^{32}\text{P}$ by mycelia

During incubation in the medium containing the  $^{32}\text{P}$  isotope, mycelia of the endophyte of E. hispidula showed linearity of absorption for approximately two minutes (Fig. 4.2). The rate of uptake from an incubating medium containing either  $\text{KH}_2^{32}\text{PO}_4$  or glucose-6-phosphate ( $^{32}\text{P}$ ) decreased rapidly from a maximum after one minute to a

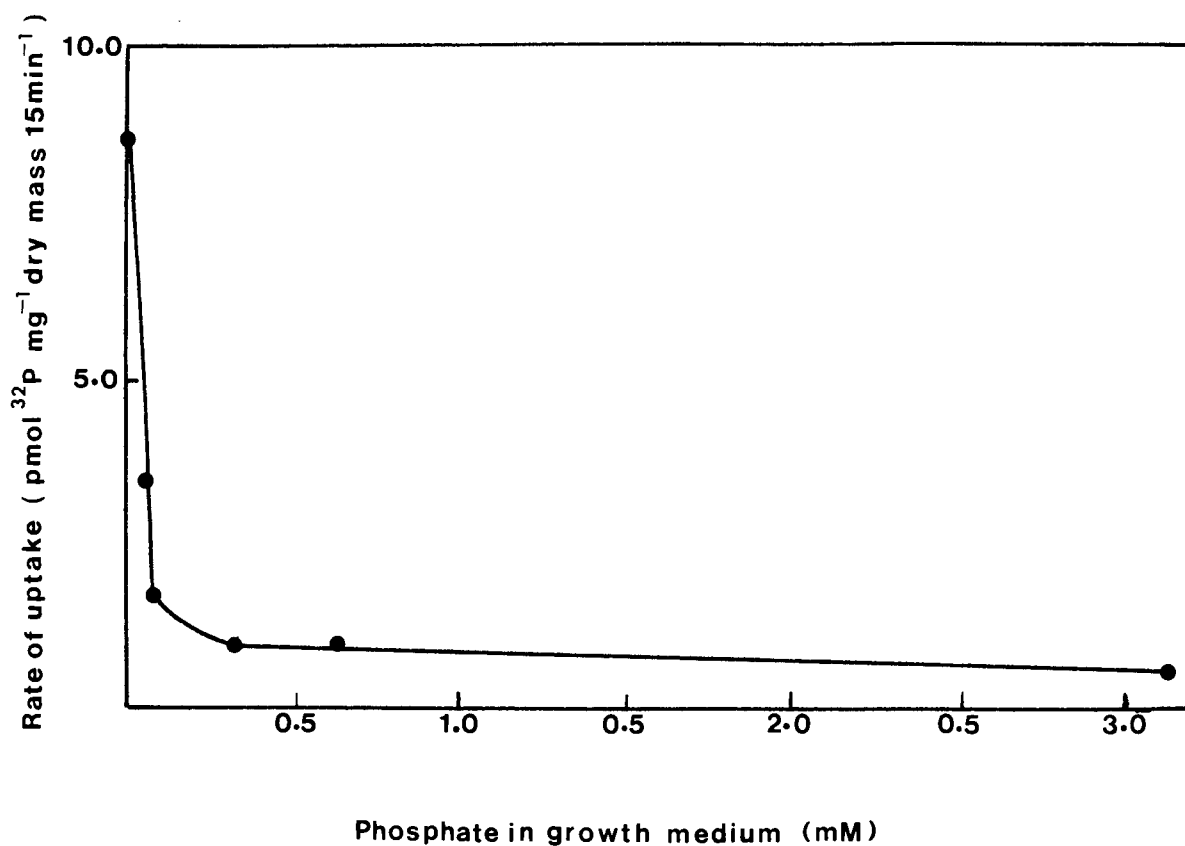


FIG. 4.1 Uptake of  $^{32}\text{P}$  from  $\text{KH}_2^{32}\text{PO}_4$  by mycelia of the endophyte of *Erica hispidula* grown for 7 d on a basal medium containing various concentrations orthophosphate ( $\text{NaH}_2\text{PO}_4$ ). Data points represent the means of 4 replicates.

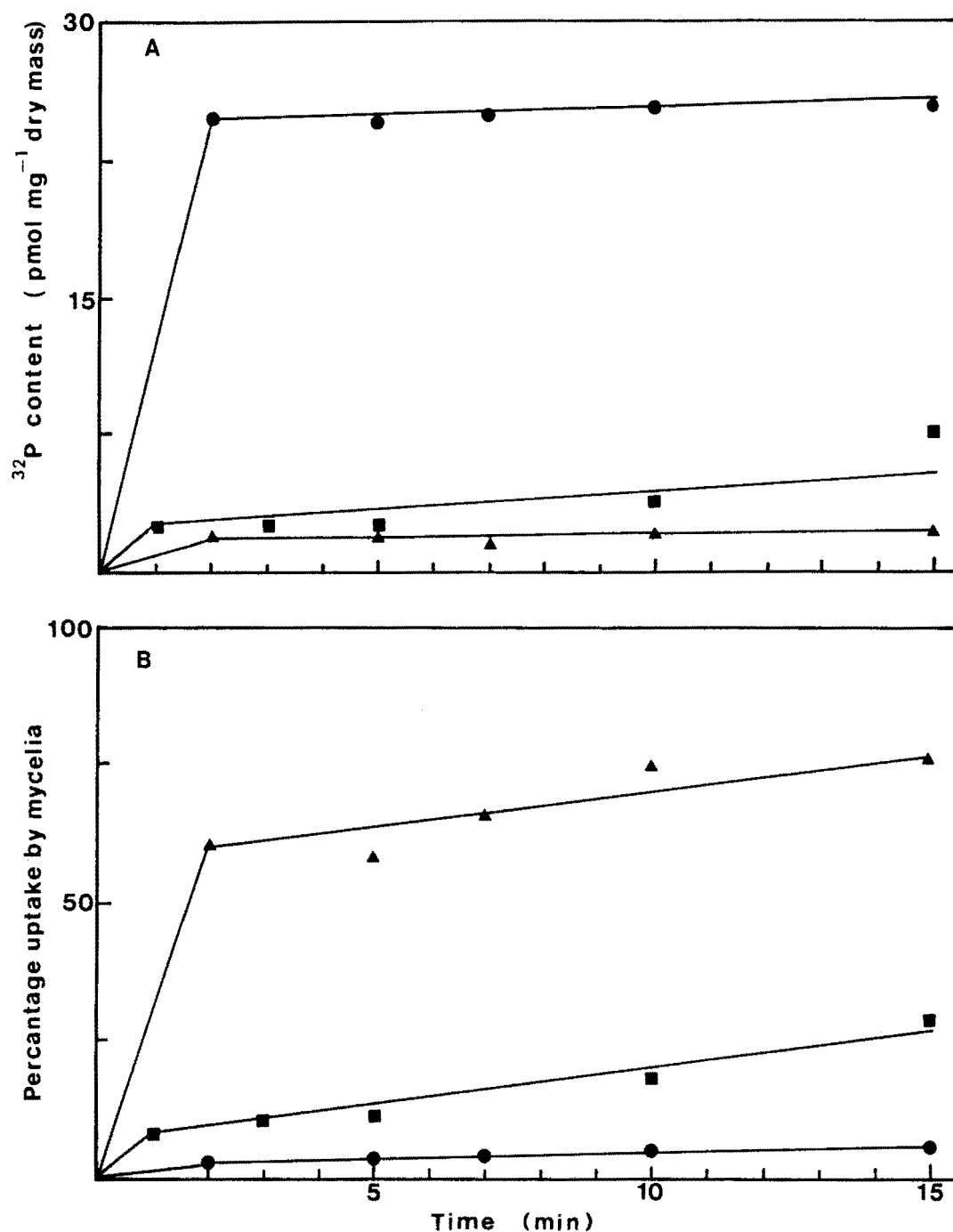
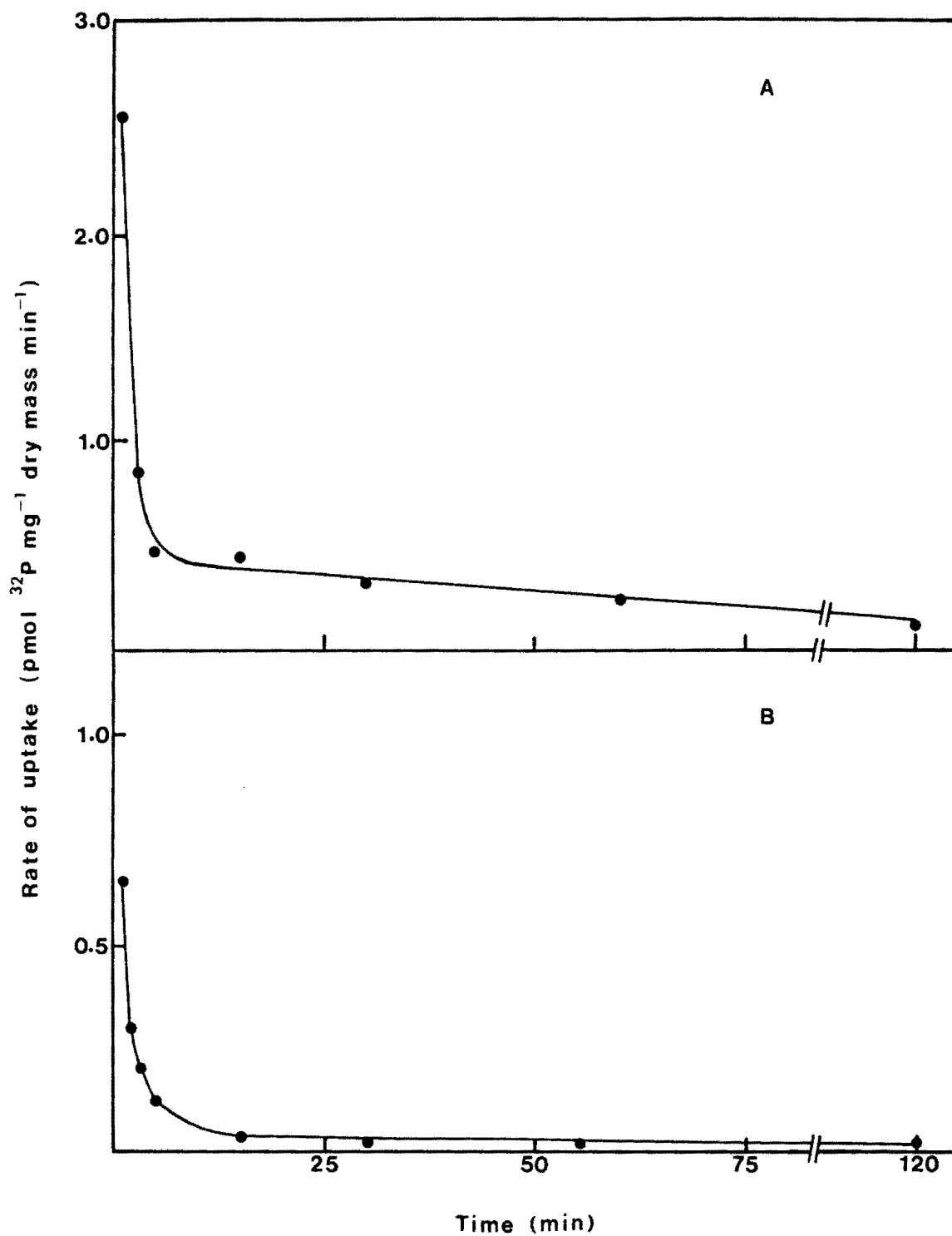


FIG. 4.2 Phosphate uptake by mycelia of the endophyte of *Erica hispidula* with time: (●—●), mycelia grown on 6 mM Pi and incubated in 500  $\mu\text{M}$  Pi, with 1.6  $\mu\text{M}$   $^{32}\text{P}$ i; (■—■), mycelia grown on 3.23 mM Pi and incubated in 0.13  $\mu\text{M}$   $^{32}\text{P}$ i; (▲—▲), mycelia grown on 0.6 mM Pi and incubated in 1  $\mu\text{M}$  Pi with  $3.2 \times 10^{-3}$   $\mu\text{M}$   $^{32}\text{P}$ i. (A) represents  $^{32}\text{P}$  content of mycelia and (B) is percentage of available  $^{32}\text{P}$  absorbed. Data points represent the means of four replicates. Pi was in the form of  $\text{KH}_2\text{PO}_4$ .





**FIG. 4.3** The rate of uptake of  $\text{KH}_2^{32}\text{PO}_4$  (A) and glucose-6- $^{32}\text{P}$  (B) over time by mycelia of the endophyte of *Erica hispidula*. Data points represent the means of 4 replicates.

steady-state rate at five minutes (Fig. 4.3). When results were expressed as a percentage of available  $^{32}\text{P}$  absorbed by mycelia against time it appears that the concentration of P in the growth medium influenced P absorption (Fig. 4.2). From these studies, the time chosen for further estimation of kinetic constants was five minutes since an incubation of one or two minutes would have caused difficulties in sampling and inaccuracies in counting.

#### 4.2.3 Efflux studies

In P uptake studies of fungi, significant efflux of the radioisotope during incubation has been observed (Lowendorff et al., 1974; Beever and Burns, 1977) and this may complicate the derivation of kinetic parameters (Neame and Richards, 1972). Efflux may occur either through some alteration of the plasmamembrane during the experimental procedure or in response to "osmotic shock" (Burns and Beever, 1977). Membrane permeability of the endophyte of E. hispidula was tested when mycelia, exposed to  $^{32}\text{P}$  orthophosphate, were incubated in a range of non-radioactive P solutions and  $^{32}\text{P}$  levels were monitored over 15 minutes. It appears that no significant efflux of  $^{32}\text{P}$  occurred with time even in salt concentrations well above those used in the kinetic studies (Table 4.1). When data were fitted to linear regression equations, all the correlation coefficients were statistically insignificant.

TABLE 4.1 The effect of incubation in solutions of increasing concentration of orthophosphate on the flux of  $^{32}\text{P}$  absorbed by 7-d-old mycelia of the endophyte of Erica hispidula.

[Phosphate] of growth medium (mM)	Time (min)	$^{32}\text{P}$ content of mycelia ( $\mu\text{mol mg}^{-1}$ dry mass) $\pm$ SE				
		Concentration of incubation medium (mM)				
0.06		0	0.06	0.6	6.0	24.0
	0	7.8 $\pm$ 2.0	7.8 $\pm$ 2.0	7.8 $\pm$ 2.0	7.8 $\pm$ 2.0	7.8 $\pm$ 2.0
	5	5.4 $\pm$ 1.1	3.3 $\pm$ 0.3	7.9 $\pm$ 2.8	8.7 $\pm$ 1.5	7.7 $\pm$ 1.8
	10	12.2 $\pm$ 1.4	4.6 $\pm$ 1.3	5.0 $\pm$ 0.6	8.5 $\pm$ 1.0	9.2 $\pm$ 0.6
	15	8.9 $\pm$ 1.7	8.5 $\pm$ 1.9	10.8 $\pm$ 1.8	15.5 $\pm$ 0.4	18.0 $\pm$ 8.0
		$r = 0.3^a$	$r = 0.2$	$r = 0.3$	$r = 0.8$	$r = 0.8$
6.0	0	17.5 $\pm$ 1.9	17.5 $\pm$ 1.9	17.5 $\pm$ 1.9	17.5 $\pm$ 1.9	17.5 $\pm$ 1.9
	5	8.2 $\pm$ 1.7	10.1 $\pm$ 1.1	10.5 $\pm$ 1.6	5.8 $\pm$ 0.2	9.4 $\pm$ 0.9
	10	15.1 $\pm$ 1.1	12.0 $\pm$ 0.7	12.8 $\pm$ 1.2	17.6 $\pm$ 3.9	23.6 $\pm$ 2.0
	15	19.3 $\pm$ 4.7	16.3 $\pm$ 2.8	19.4 $\pm$ 1.4	25.1 $\pm$ 2.5	22.3 $\pm$ 2.2
		$r = 0.04^a$	$r = 0.03$	$r = 0.3$	$r = 0.6$	$r = 0.6$

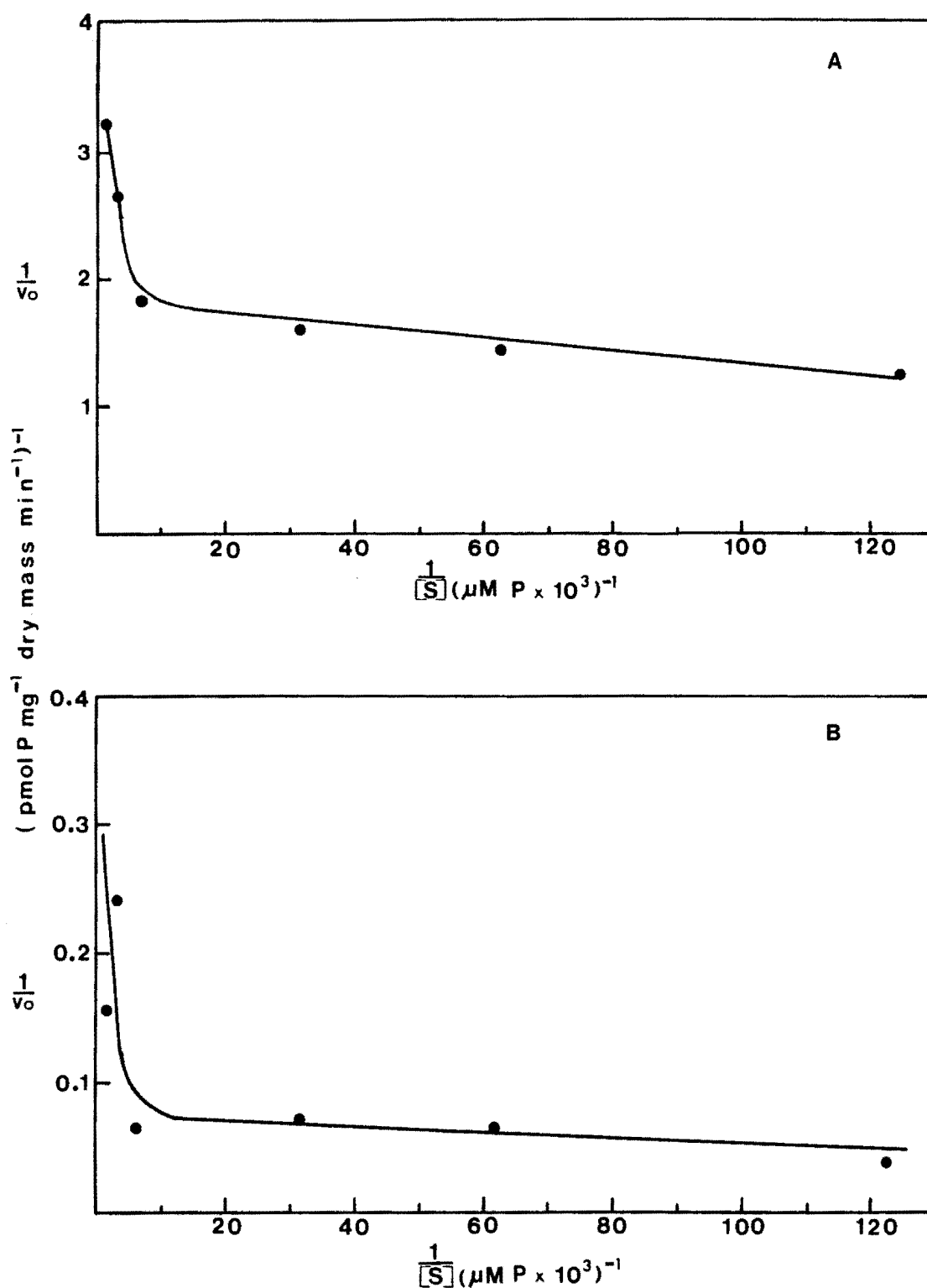
<sup>a</sup> Correlation co-efficients obtained when data were fitted to straight line regression equations.

#### 4.2.4 Preliminary experiments on the rate of uptake by mycelia in relation to P concentration.

When mycelia were incubated in solutions containing a constant concentration of  $^{32}\text{P}$  but a wide range of non-radioactive P concentrations, uptake rate (as represented by  $^{32}\text{P}$  uptake) formed a curvilinear relationship with P concentration when results were transformed to double-reciprocal (Lineweaver-Burk) plots (Fig. 4.4). The transformation in Fig. 4.4 indicated the operation of a dual-uptake system but kinetic parameters (i.e.  $K_m$  and  $V_{max}$ ) were not determined from these results since the ratio of  $^{32}\text{P}$  to non-radioactive P in the incubation medium had not been kept constant.

#### 4.2.5 The effect of pH on P uptake kinetics

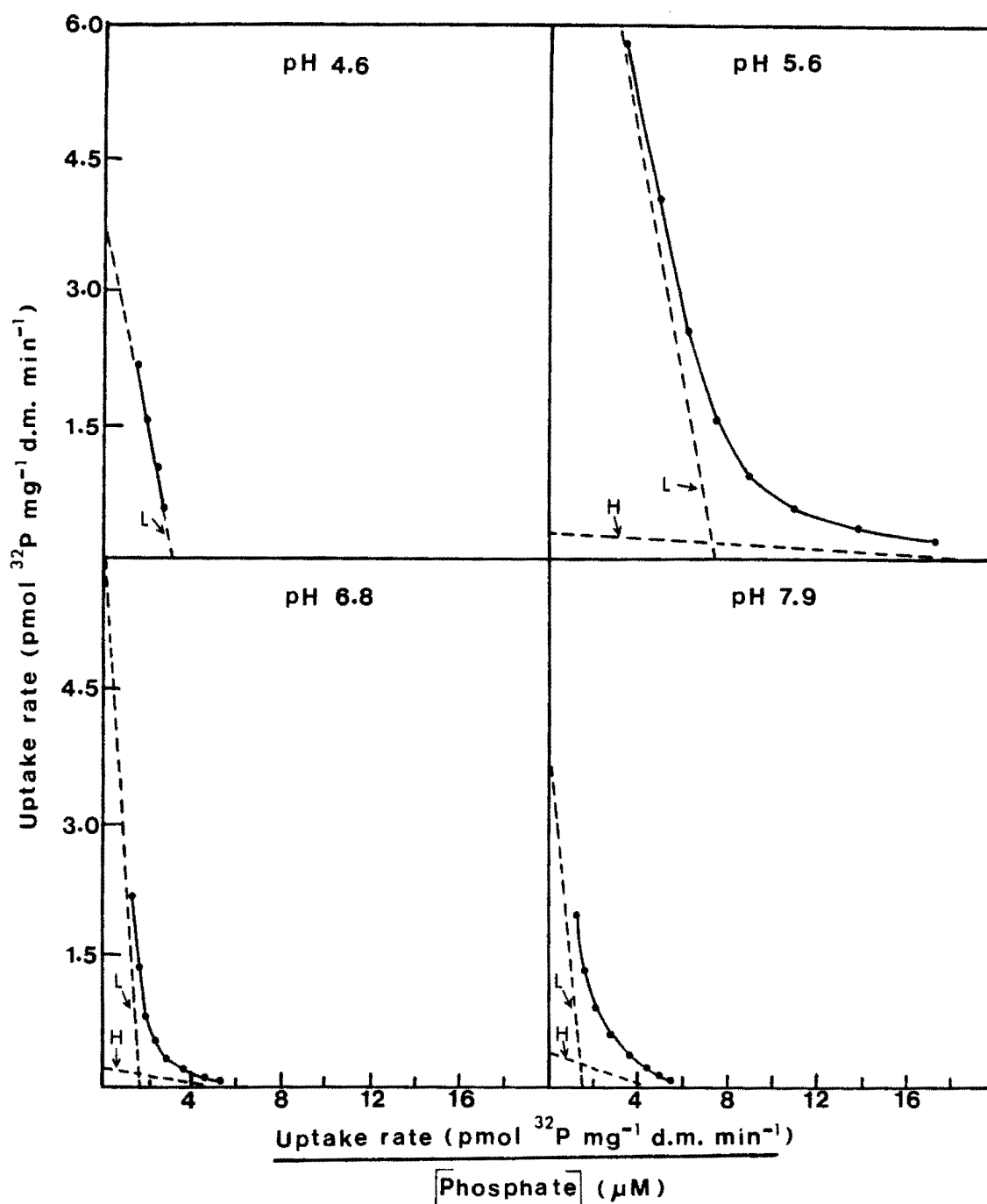
Since the pH of the external medium may influence uptake rates and thus the values of kinetic parameters (Lowendorff *et al.*, 1974), it was necessary to determine optimal pH conditions for the estimation of kinetic parameters for the endophyte. When mycelia were incubated in a wide range of P orthophosphate (0.01  $\mu\text{M}$  to 1.68  $\mu\text{M}$ ) dissolved in buffers of different pH values (4.6, 5.6, 6.8 and 7.9), transformations to Hofstee plots indicated curvilinear responses at pH 5.6, 6.8 and 7.9 but not at 4.6 (Fig. 4.5). The parameter estimates of the high-affinity system at pH 4.6 were not determinable. At pH 4.6, uptake rates from the four lower concentrations differed slightly so that the



**FIG.4.4** Double reciprocal plots of rate of uptake  $1/v$  vs concentration by mycelia of the endophyte of *Erica hispidula* incubated in either  $20 \mu\text{Ci dm}^{-3} \text{KH}_2^{32}\text{PO}_4$  (A) or glucose-6- $^{32}\text{P}$  (B) with various concentrations orthophosphate ( $\text{KH}_2\text{PO}_4$ ). Data points represent the means of 4 replicates.

double-hyperbola curve fit programme fitted the data to a horizontal straight line with a  $V_{max}(HA)$  value approaching zero and an indeterminable  $K_m(HA)$  value. However, the close correspondence between the line representing the sum of the two systems and that representing the low-affinity system (Fig. 4.5) suggests that the high-affinity system contributed a negligible amount to total uptake at pH 4.6.

A comparison of  $K_m$  values of the low-affinity system with pH showed the lowest value at pH 4.6 with the highest at pH 6.8 (Table 4.2). The  $V_{max}(LA)$  was also lowest at pH 4.6 but highest at pH 5.6. The  $K_m(HA)$  values of the high affinity systems were low but increased nine-fold from pH 5.6 to 7.9, whereas  $V_{max}(HA)$  values showed small variations with pH with no definite trend. These kinetic constants were calculated from uptake rates which were the means of four replicates and replication to some extent, nullified any inaccuracies due to experimental error. It was found that the BASIC computer programme was unable to calculate satisfactory kinetic constants when each set of replicates, with its limited number of concentrations variables, was treated as a separate experiment. Thus it was not possible to establish the statistical significance of the variation between kinetic constants with pH.



**FIG. 4.5** Hofstee plots from uptake data obtained over a wide concentration range <sup>32</sup>P (0.01 μM to 1.68 μM) buffered to different pH values, by mycelia of the endophyte of *Erica hispidula* grown for 7 d on 6 mM orthophosphate. The high-affinity and low-affinity systems are shown by the dotted lines marked H and L respectively. The solid lines are the calculated sums of the 2 systems. Original data points represented the means of 3 or 4 replicates.

**TABLE 4.2** The influence of pH on the dual-system kinetic parameter estimates of the P uptake systems of mycelia of the endophyte of Erica hispidula grown on 6 mM P for 7 d.

pH	System			
	<u>Low-affinity</u>		<u>High-affinity</u>	
	$K_m$ ( $\mu\text{M}$ )	$V_{max}$ ( $\text{pmol mg}^{-1}\text{d.m.min}^{-1}$ )	$K_m$ ( $\mu\text{M}$ )	$V_{max}$ ( $\text{pmol mg}^{-1}\text{d.m.min}^{-1}$ )
4.6	1.14	3.68	-	-
5.6	1.39	10.22	0.01	0.26
6.8	3.68	6.32	0.04	0.18
7.9	2.59	4.05	0.09	0.40

- ; signifies not determinable (see text)



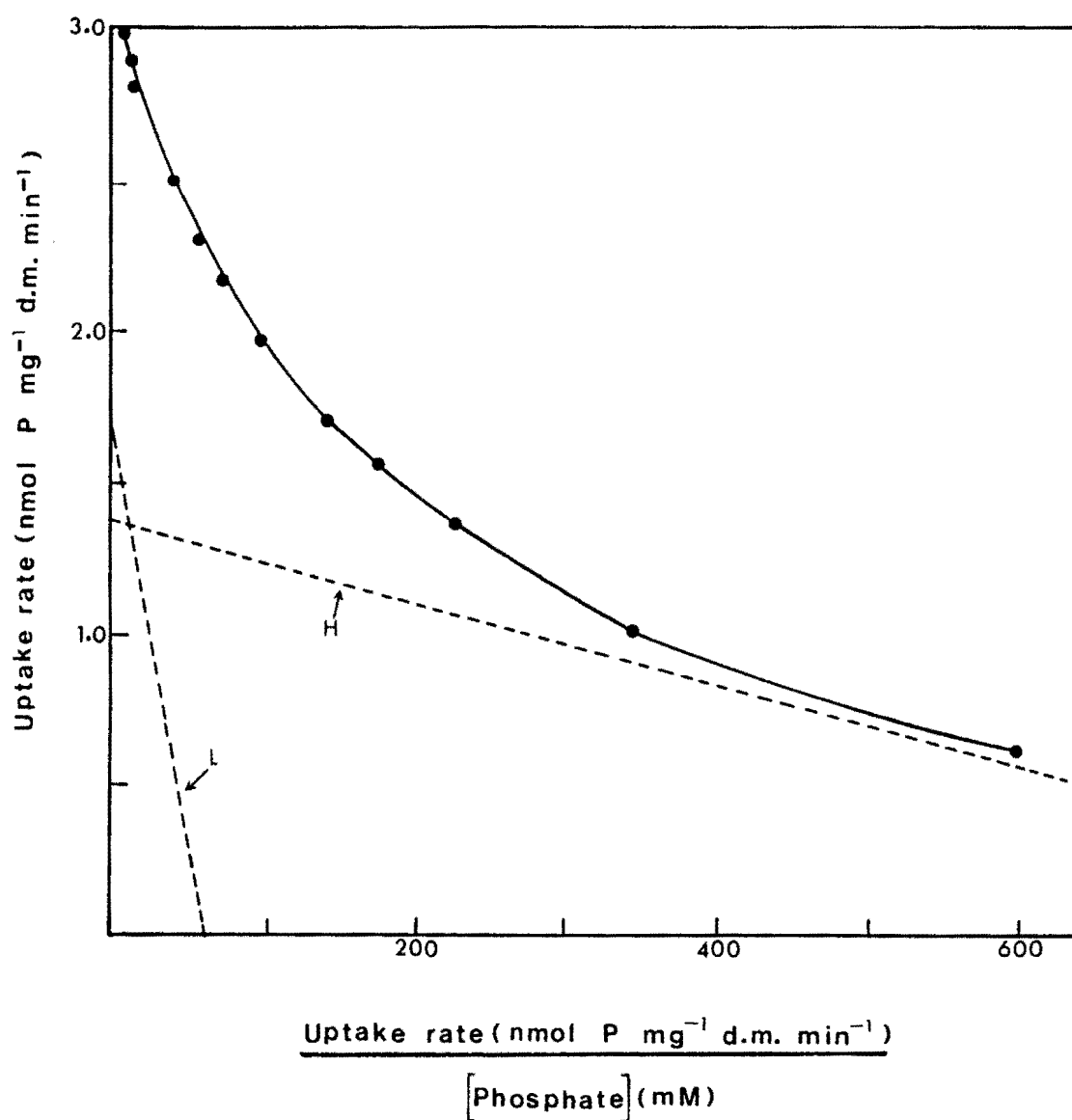
#### 4.2.6 The effect of a metabolic inhibitor on uptake

2,4-Dinitrophenol (DNP) inhibits the oxidative phosphorylation of ATP synthesis and is commonly used as an inhibitor of active ion transport across membranes. The kinetic parameters of the low-affinity system of mycelia growing in high P (6 mM) media were estimated at pH 5.6 in the presence and absence of 2,4 dinitrophenol. Without DNP in the medium the  $V_{max}$  value was  $9.76 \text{ pmol } ^{32}\text{P mg}^{-1} \text{ dry mass min}^{-1}$  and was reduced by 90% to  $1.0 \text{ pmol } ^{32}\text{P mg}^{-1} \text{ dry mass min}^{-1}$  when DNP was present. The addition of DNP caused a small increase in the  $K_m$  value from 2.0 to 2.9  $\mu\text{M P}$ .

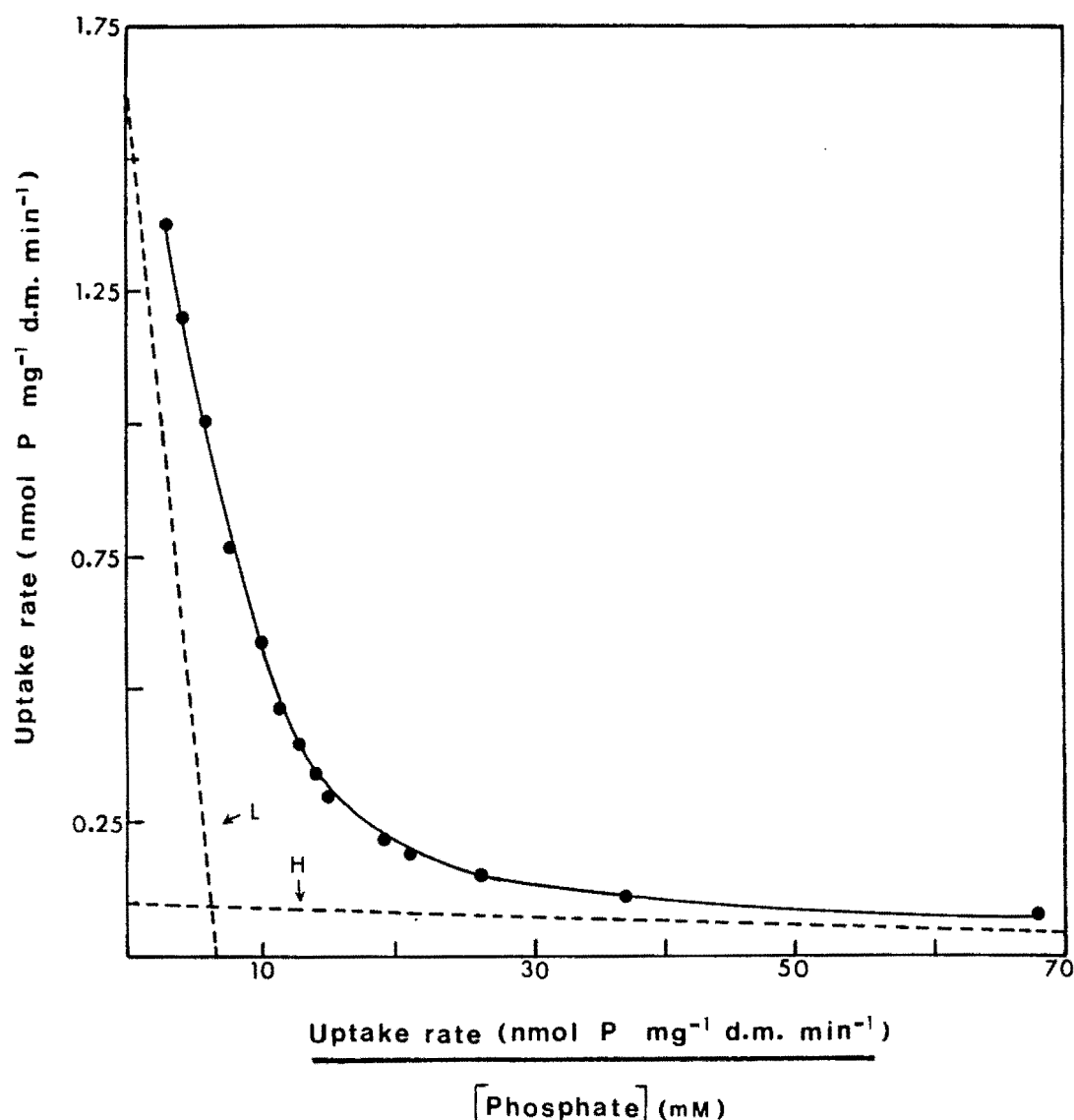
#### 4.2.7 The kinetic parameters of dual-system uptake in high P and low P-fed mycelia

Further experiments were designed to determine more precise estimates of the kinetic parameters of high-and low-affinity systems in the endophyte of E. hispidula at pH 5.6. To allow for the possibility that kinetic parameters "might vary according to the physiological status of the fungus" (Burns and Beever, 1977), mycelia were grown in a basal medium containing either high (6 mM) or low (0.06 mM) levels of orthophosphate ( $\text{KH}_2\text{PO}_4$ ). Seven days after inoculation, mycelia were incubated for five minutes in a wide range of orthophosphate solutions (1  $\mu\text{M}$  to 0.5 mM) and the levels of  $^{32}\text{P}$  absorbed by mycelia were measured. The range of incubation concentrations used was assessed from preliminary results (Fig. 4.4).

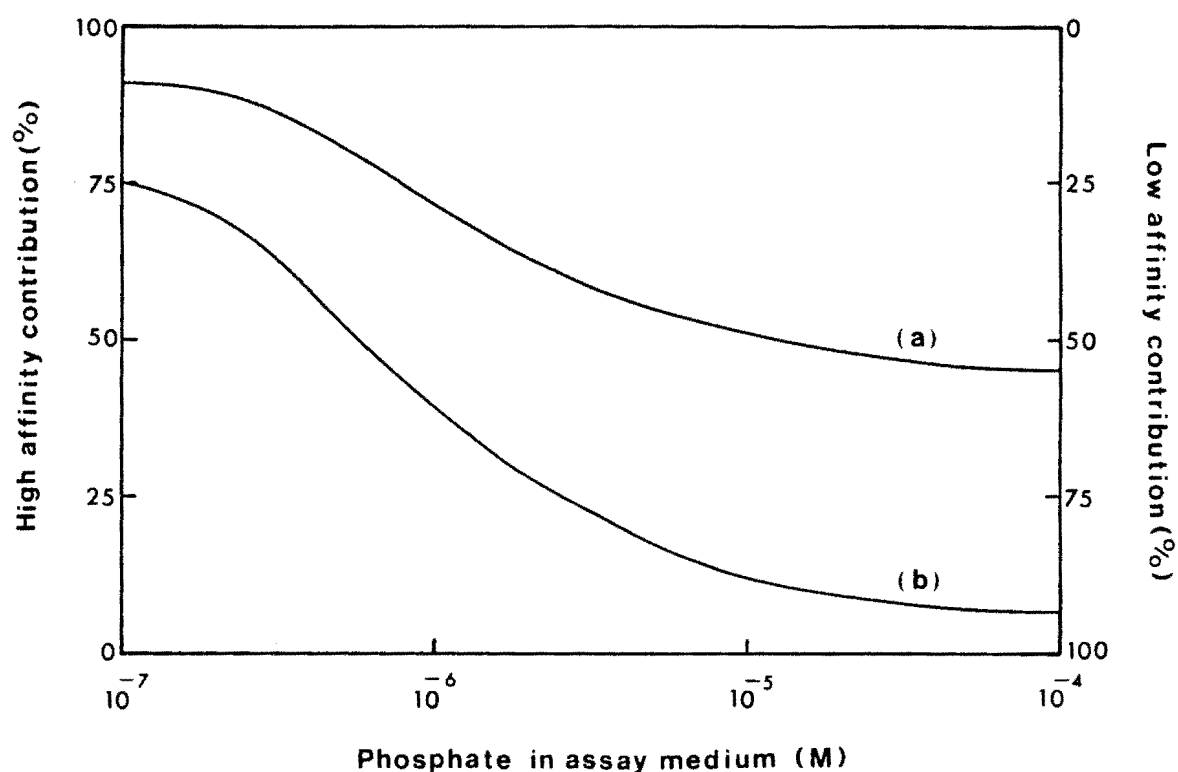
The uptake rates of both high and low P-fed mycelia showed a distinct curvilinear relationship with concentration when displayed on a Hofstee plot (Figs. 4.6 and 4.7). Partition into subsets occurred between 40  $\mu\text{M}$  and 60  $\mu\text{M}$  P. Table 4.3 presents the parameter values derived from the Hofstee plots. These values are based on the total amount of P available for uptake, both radioactive and non-radioactive. In the low-affinity systems, the  $V_{max}(\text{LA})$  estimates of high P- and low P-fed mycelia were similar, whereas the  $K_m(\text{LA})$  estimate of high P-fed mycelia was 5.5 times lower than that of low P-fed mycelia. In the high-affinity systems, the  $K_m(\text{HA})$  values of high P- and low P-fed mycelia were similar, whereas the  $V_{max}(\text{HA})$  value of high P-fed mycelia was 14 times greater than that of low P-fed mycelia. The relative contribution of each system to total uptake over the entire concentration range of 1  $\mu\text{M}$  to 0.5 mM P is shown in Fig. 4.8. In high P-fed mycelia, the two systems contributed equally to uptake at 0.1 mM P whereas in low P-fed mycelia equal contribution occurred at 0.05 mM P. At the highest concentrations (i.e. above  $10^{-5}$  M) the percentage of the low-affinity system to total uptake was 46% in high P-fed mycelia but 93% in low P-fed mycelia. At the lowest concentrations the proportion of the low-affinity system to total uptake was 8% in high P-fed mycelia but 25% in low P-fed mycelia.



**FIG. 4.6** Hofstee plot for data obtained over a wide P concentration range (1  $\mu$ M to 0.5 mM) from mycelia of the endophyte of *Erica hispidula* grown for 7 d on 6 mM orthophosphate. The high-affinity system ( $K_m$ , 1.5  $\mu$ M;  $V_{max}$  1.4 nmol P mg<sup>-1</sup>d.m. min<sup>-1</sup>) is represented by the dotted line, H. The low affinity system ( $K_m$ , 27.5  $\mu$ M;  $V_{max}$ , 1.7 nmol P mg<sup>-1</sup>d.m.min<sup>-1</sup>) is represented by the dotted line L. The solid line is the calculated sum of the two systems. Results are the means of calculated values obtained from 4 replicates.



**FIG. 4.7** Hofstee plot for data obtained over a wide concentration range (1  $\mu\text{M}$  to 0.5 mM) from mycelia of the endophyte of *Erica hispidula* grown for 7 days on 0.06 mM orthophosphate. The high affinity system ( $K_m$ , 0.9  $\mu\text{M}$ ;  $V_{max}$ , 0.1 nmol P mg<sup>-1</sup> d.m.min<sup>-1</sup>) is shown by the dotted line H. The low affinity system ( $K_m$ , 151.5  $\mu\text{M}$ ;  $V_{max}$ , 1.6 nmol P mg<sup>-1</sup> d.m.min<sup>-1</sup>) is shown by the dotted line L. The solid line is the calculated sum of the two systems. Results are the means of calculated values obtained from 4 replicates.



**FIG. 4.8** Diagram showing the relative contributions of the high- and low-affinity systems in 6 mM -fed (a) and 0.06 mM -fed (b) mycelia to uptake over a wide P concentration range. Curves were calculated from values shown in Figs. 4.6 and 4.7. Phosphate concentration in the assay medium is represented on a logarithmic scale.

TABLE 4.3    Summary of dual-system kinetic parameter estimates of the P uptake systems of high P- and low P-fed mycelia of the endophyte of Erica hispidula.<sup>a</sup>

System	0.06 mM mycelia	6 mM mycelia	$t^c$ value	P
<u>Low-affinity</u>				
$K_m$ (μM) <sup>±</sup> SE	151.50 <sup>±</sup> 23.5	27.48 <sup>±</sup> 8.7	5.43	<0.01
$V_{max}$ (μmol P g <sup>-1</sup> d.m. min <sup>-1</sup> ) <sup>±</sup> SE <sup>b</sup>	1.63 <sup>±</sup> 0.1	1.68 <sup>±</sup> 0.3	0.12	NS
<u>High-affinity</u>				
$K_m$ (μM) <sup>±</sup> SE	0.88 <sup>±</sup> 0.4	1.53 <sup>±</sup> 0.8	2.17	NS
$V_{max}$ (μmol P g <sup>-1</sup> d.m. min <sup>-1</sup> ) <sup>±</sup> SE <sup>b</sup>	0.10 <sup>±</sup> 0.2	1.38 <sup>±</sup> 0.1	9.14	<0.001

<sup>a</sup> Values are based on four replicates treated as four separate experiments

<sup>b</sup> Values converted from nmol mg<sup>-1</sup>d.m.min<sup>-1</sup> to allow for comparisons with values in Table 1.1

<sup>c</sup> degrees of freedom = 6

#### 4.3 DISCUSSION

The uptake kinetics of a number of ions in both higher plants (Epstein and Elzam, 1963; Epstein, 1966) and fungi (Beever and Burns, 1980) has been based upon a dual uptake system which assumes that two systems, of different kinetic parameters, operate simultaneously with each contributing to uptake at all concentrations. Thus uptake at any one concentration is the sum of two Michaelis-Menten equations. When uptake rates in relation to substrate concentration are transformed dual-system uptake will form a continuous and curvilinear plot (Nissen, 1973; 1974). However, the dual-system interpretation is one of several which can be applied to uptake data. Nissen (1973; 1974) has shown that ionic uptake data (including that of P uptake) from higher plants, which had originally been analysed to show a dual uptake system could be reinterpreted with more detailed and precise data to indicate a multiphasic system of uptake. Transformations of such data show discontinuous curves, indicating transitions from one phase to another. Other alternatives to dual uptake mechanisms which have similar kinetic data to those of dual systems, have been proposed (Nissen, 1974; Borst-Pauwels, 1976). Borst-Pauwels (1976) established statistical criteria for distinguishing dual uptake systems from other mechanisms such as non-carrier transport, two-site carrier transport and single-site transport with multiple binding sites. However, these

methods require detailed and very accurate data and Beever and Burns (1980) have cited direct evidence for the existence of dual uptake systems. This evidence includes mutants of N. crassa containing only one system and also the differential response of the systems in Saccharomyces cerevisiae Meyen ex Hansen to external ions.

Preliminary experiments on the endophyte of E. hispidula indicated the possible operation of a dual system of uptake which was further investigated by using the statistical procedures of Burns and Tucker (1977). Beever and Burns (1980) have indicated the operation of dual-system uptake in ectomycorrhizas and soil saprotrophs (Table 1.1, p.16) and these values can be compared with those obtained for low P-fed mycelia of the endophyte of E. hispidula. The  $K_m(\text{HA})$  value ( $0.88 \mu\text{M}$ ) of the ericoid endophyte is between two and eleven times lower than any of the values listed in Table 1.1. The  $V_{max}(\text{HA})$  value ( $0.1 \text{ nmol g}^{-1} \text{ d.m. min}^{-1}$ ), although ten times higher than those of intact ectomycorrhizas is twenty times lower than those of N. crassa and Aspergillus nidulans (Eidam) Winter. Similarly the  $K_m(\text{LA})$  value ( $151.5 \mu\text{M}$ ) of the endophyte is between seven and twenty-one times lower than those of ectomycorrhizas and two and four times lower than that of N. crassa and A. nidulans respectively. The  $V_{max}(\text{LA})$  value ( $1.6 \text{ nmol g}^{-1} \text{ d.m. min}^{-1}$ ) of the endophyte, although between six and thirty times higher than those of ectomycorrhizas



comparison of the other kinetic constants of the high affinity and low-affinity systems does show differences between the two organisms. The  $V_{max}(LA)$  and  $V_{max}(HA)$  values of low P-fed germlings of N. crassa were significantly higher than corresponding values of high P-fed germlings and the  $K_m(LA)$  value of low P-fed germlings was significantly lower than that of high P-fed germlings. It was concluded that low P-fed germlings achieved a greater efficiency at taking up P from dilute solutions by increasing the efficiency of their low-affinity system. In contrast, there was no clear enhancement of the efficiency of the low-affinity system of low P-fed mycelia of the endophyte. Under conditions of limited P availability, there was a repression of the high-affinity system with the endophyte relying more on its low-affinity system for uptake. The high P-fed mycelia "indulged" in high-affinity uptake even at high concentrations of P in the external medium (54% of total uptake at 0.5 mM P). If the adaptive importance of the low-affinity system lies in its reduced energy demands on the cell (Beever and Burns, 1980), it would appear that the differences in the uptake kinetics between high P- and low P-fed mycelia of the endophyte might be explained by the importance of P in the energetics of cell metabolism.

These investigations on ericoid mycorrhizas, show that although the high-affinity system appears to be the dominant

one at low phosphorus concentrations, the low-affinity system plays a significant role in phosphorus uptake, particularly when the organism is growing in a low phosphorus environment. This observation is confirmed by the pH studies (Fig. 4.5; Table 4.2) in which low-affinity uptake was detectable at external concentrations below 2  $\mu\text{M}$  P. Levels of available P in many soils may be as low as 0.01  $\mu\text{M}$  to 2  $\mu\text{M}$  (Woolhouse, 1975) and the levels of resin-extractable P in fynbos soils were shown to vary between 13  $\mu\text{M}$  and 81  $\mu\text{M}$  P (Mitchell, Brown and Jongens-Roberts, 1984).

Phosphate uptake by fungi is carrier-mediated and accompanied by ATP-dependent proton co-transport against an electrochemical gradient (Woolhouse, 1975; Beever and Burns, 1980). The results of this investigation confirm that when ATP synthesis is interrupted by 0.5 mM DNP, an inhibitor of oxidative phosphorylation, the rate of uptake by mycelia of the endophyte of E. hispidula is severely reduced. Optimum uptake of P usually occurs at pH values around 5 (see Goodman and Rothstein, 1957) when  $\text{H}_2\text{PO}_4^-$  is the predominant P ion. When the uptake systems of mycelia were operating at different pH values, optimal activity of the low-affinity and possibly high-affinity systems occurred at pH 5.6. In both systems of the endophyte of E. hispidula, there was a trend towards an increase in  $K_m$  although only three-fold in the low-affinity and nine-fold in the high-affinity system. The  $V_{max}$  values followed no particular

pattern within each system but the ratio of  $V_{max}(LA)$ :  $V_{max}(HA)$  decreased from pH 4.6 to 7.9. There thus appeared to be a relationship between pH and the contribution of the high-affinity system to uptake with pH. In pH studies on N. crassa, the effect of pH on the high-affinity system was negligible whereas the low-affinity one was strongly affected;  $V_{max}(LA)$  values remained fairly constant but  $K_m(LA)$  values increased 300-fold between pH 4.0 and 7.3 (Lowendorff et al., 1974, 1975). These results were not explained in terms of two completely separate transport systems (one with a low  $K_m$  and low pH optimum and the other with a high  $K_m$  and high pH optimum) and did not support the hypothesis that only  $H_2PO_4^-$  and not  $HPO_4^{2-}$  was the substrate for the transport system. A model was proposed in which  $H^+$  (or  $OH^-$ ) served as modifiers of the uptake systems by altering their affinities for the substrate (either  $H_2PO_4^-$  or  $HPO_4^{2-}$ ).

The absorption of P from solutions, similar in concentration to those of the soil, has been shown to be linear with time in beech mycorrhizas with a rate decrease after only very long periods of time (Harley, 1969). At higher concentrations of external P, however, uptake was not linear with time. Cress et al. (1979) established that at 1 and 60  $\mu M$  P, uptake by tomato roots infected with VA mycorrhizas was linear for up to 25 min. Linearity of absorption by mycelia of the endophyte of E. hispidula occurred within one

to two minutes but the rate of uptake declined dramatically between 0 and 5 min (Fig. 4.3). After 5 minutes a steady-state decline occurred which could not be attributed to any efflux of phosphate (Table 4.1). These results indicate that after an initial rapid influx of P, the endophyte adjusts its uptake rate so that P is absorbed only according to requirements, especially when the phosphate ion is abundant. The pattern of uptake appears to be dependent upon both external and internal phosphorus levels (Fig. 4.1). N. crassa appears to exert a similar tight control over the activity of its uptake systems to maintain a constancy of P uptake over a wide P concentration range (Beever and Burns, 1977). The constancy of uptake and the maintenance of a constant internal P content was adequately explained by changes in the kinetic constants of the high- and low-affinity uptake systems.

This investigation indicates that the ericoid endophyte may well improve the P status of its host through its ability to use an energetically inexpensive low-affinity uptake system at very low soil phosphorus concentrations and to bring about rapid adjustments in uptake in response to both soil and host P levels.

## CHAPTER 5

### THE IDENTIFICATION, EXTRACTION AND FRACTIONATION OF POLYPHOSPHATES AND PHYTIC ACID

#### 5.1 INTRODUCTION

Ultrastructural analyses of ericoid mycorrhizas indicate that the P absorbed by extracellular hyphae may be transformed into electron-dense, osmiophilic, vacuolar granules of polyphosphate (polyP) which in dual culture only persist in the extracellular mycelium (Bonfante-Fasola and Gianinazzi-Pearson, 1982). In natural ericoid mycorrhizas, polyP granules have been found in both the intra- and extracellular mycelium (Bonfante-Fasola et al., 1981). It appears therefore that the synthesis of polyP is dependent on the nutrient and metabolic status of the endophyte in relation to the host and the external environment. Apart from ultrastructural studies no information is available on the role of polyP either in the P metabolism of ericoid mycorrhizas or its function as a potential storage form of P. PolyP may be of fundamental importance in the survival of ericaceous plants especially when situated under conditions of low phosphorus status. This chapter describes an investigation into the identification, characterisation and estimation of polyP in cultured endophytes and synthesized mycorrhizal and non-mycorrhizal seedlings. Experiments involving the fractionation of phosphorus were designed to establish the

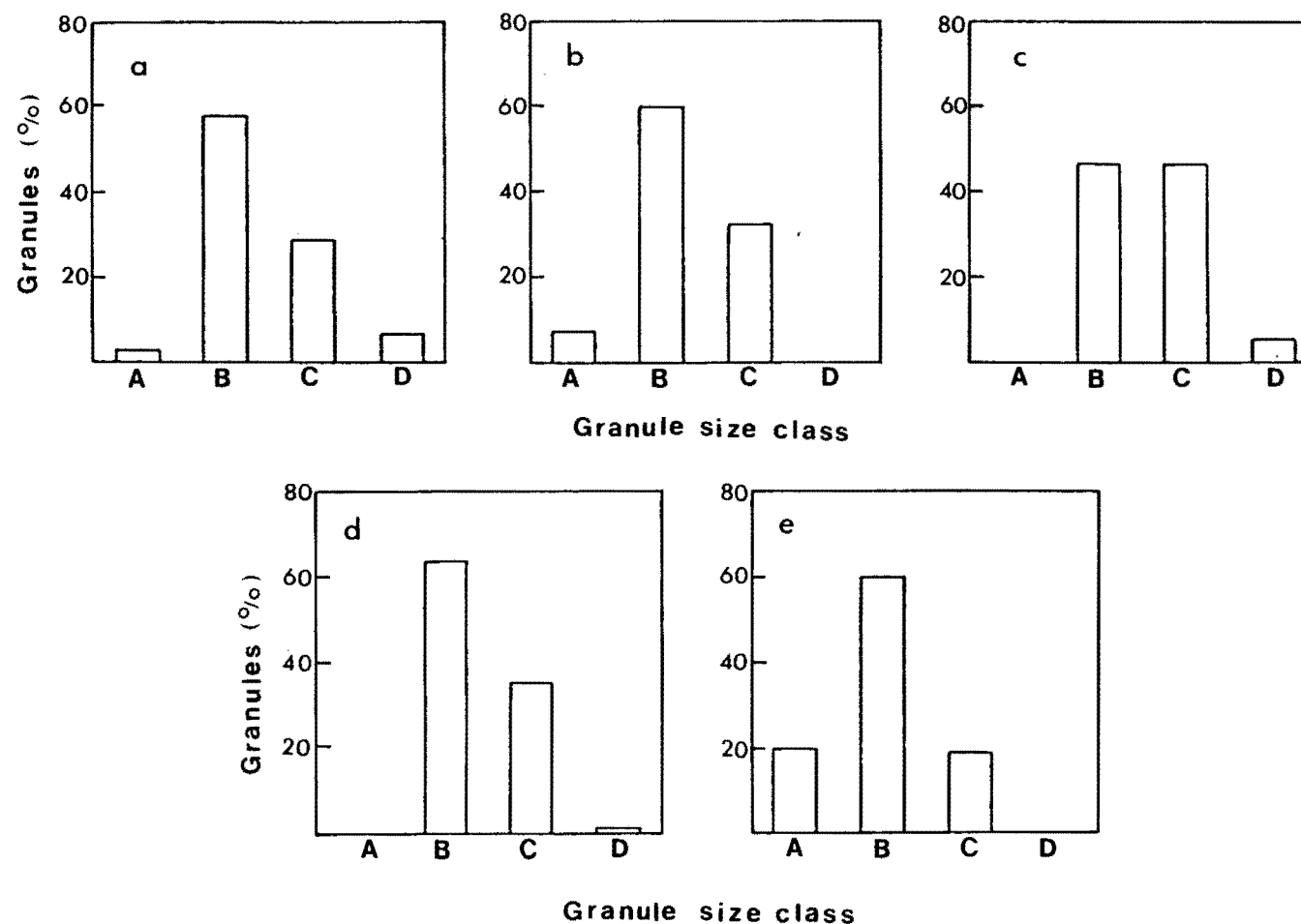
potential of isolated endophytes to store excess P as polyP and to confirm the importance of polyP in the P cycle of the fungus. The possibility that the endophyte could synthesize phytic acid as a storage form was also tested. Part of this chapter was published as a paper entitled 'The Characterization and Estimation of Polyphosphates in Endomycorrhizas of the Ericaceae' (C.J. Straker and D.T. Mitchell) in The New Phytologist (1985), 99, 431 - 440.

## 5.2 RESULTS

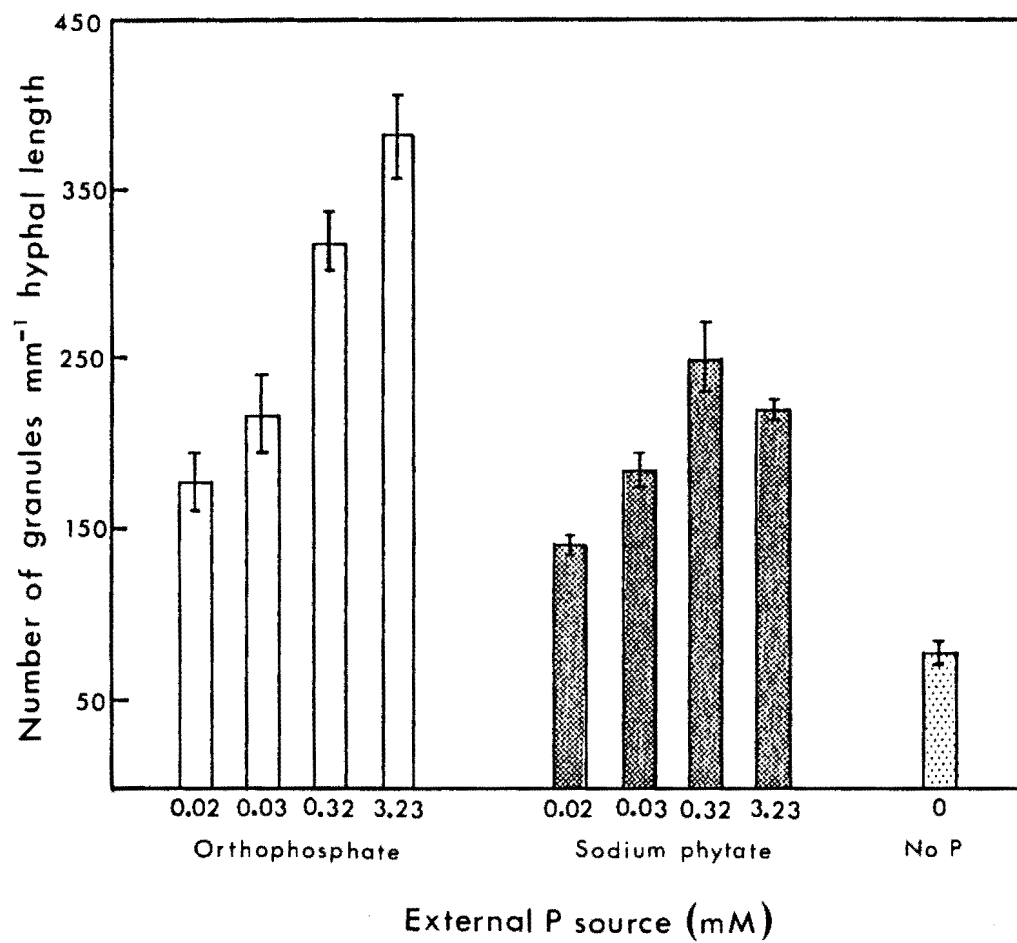
### 5.2.1 Cytochemical observations of polyP granules

A positive metachromatic reaction was obtained when hyphae were stained with toluidine blue at pH 1.0 and granules contained within vacuoles were readily observed in young hyphae (Fig. 5.1). In older hyphae, vacuoles were larger and the granules were not easily identifiable. Figure 5.2 shows the range of size classes for five different endophytes. Correspondence Analysis revealed that granules of E. mauritanica were smaller than those of the other endophytes under similar growing conditions. When material was stained with lead nitrate followed by ammonium sulphide, granules similar in size, frequency and distribution to metachromatic granules were stained black.

Numbers of metachromatic granules could be used as a measure of concentration of polyP and Figure 5.3 shows their



**FIG. 5.2** The percentage of metachromatic granules in relation to different size classes in 10-d-old mycelia of endophytes isolated from root systems of (a) *Calluna vulgaris* (b) *Vaccinium macrocarpon*, (c) *Rhododendron ponticum*, (d) *Erica hispidula*, (e) *Erica mauritanica*, grown on basal medium and 3.23 mM sodium phytate. (Size classes are based on diameter of granules as follows: A, <0.5  $\mu\text{m}$ ; B, 0.5  $\mu\text{m}$  to 1.0  $\mu\text{m}$ ; C, 1.0  $\mu\text{m}$  to 1.5  $\mu\text{m}$ ; D, >1.5  $\mu\text{m}$ )



**FIG. 5.3** Numbers of metachromatic granules in endophyte of *Vaccinium macrocarpon* grown for 14 d in liquid culture with various sources of P. Vertical bars represent twice SE.



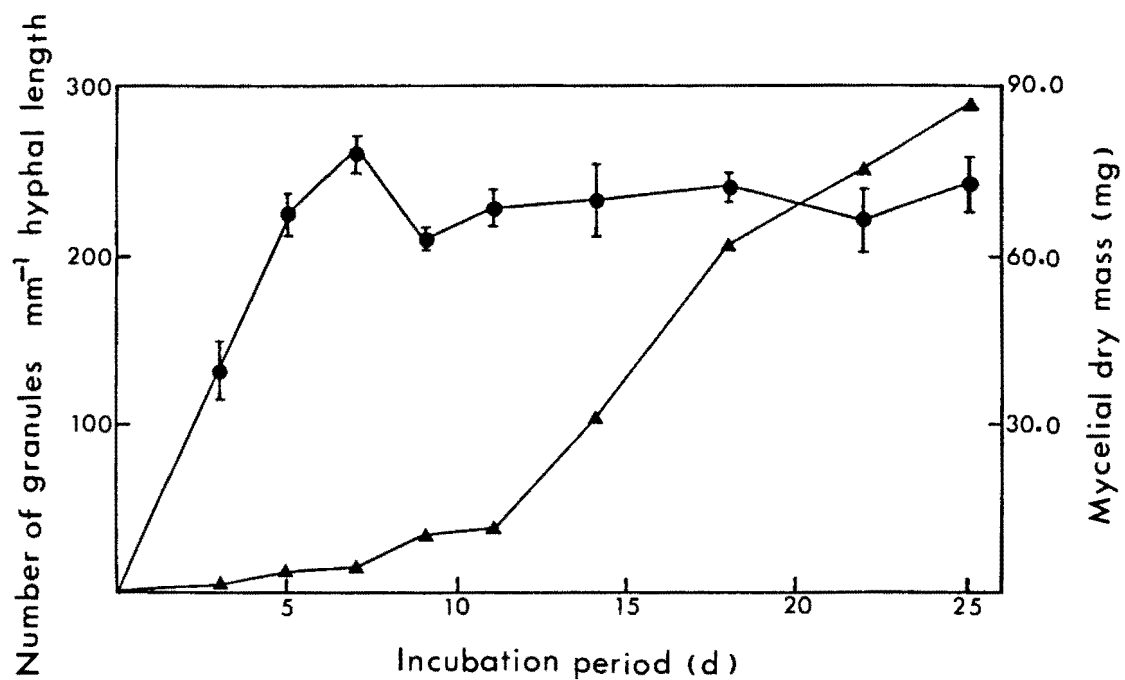
relationship to the external concentration of phosphorus in the medium. When the P source was orthophosphate, there was a direct power curve relationship between these variables ( $y = 155.6x^{0.11}$  with  $r^2 = 0.94$ ). However, when P was supplied in an organic form, granule numbers increased up to an external concentration of 0.32 mM but thereafter they declined. Granules in hyphae during the growth of the endophyte in culture accumulated rapidly during the lag phase of growth (Fig. 5.4).

#### 5.2.2. Polyacrylamide gel electrophoresis for separation of nucleic acid-polyP co-precipitates

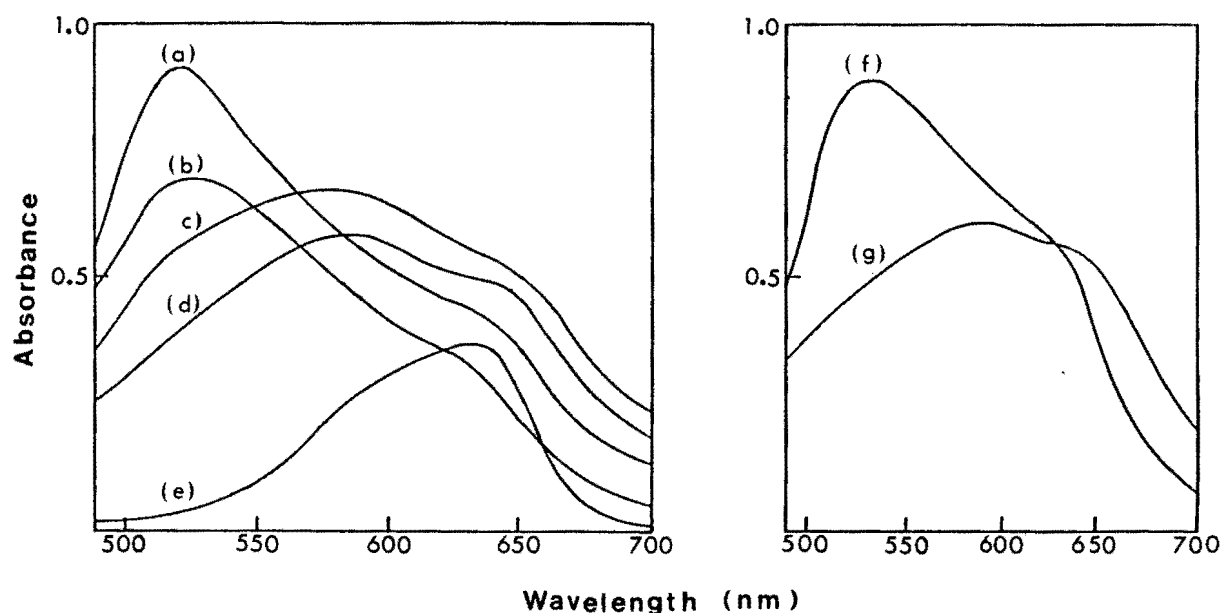
The pink staining bands obtained from mycelial extracts of a South African and European isolate as well as commercial polyP (sodium phosphate glass Type 65) have absorption maxima at 530 nm ( $\gamma$ -metachromasy) (Fig. 5.5). Blue metachromatic gel bands from mycelial extracts and commercial RNA showed a shift to an absorption maximum of 580 nm ( $\beta$ -metachromasy) (Fig. 5.5). The  $\gamma$ -metachromasy of the pink bands produced absorption peaks at 523 nm whereas the major blue bands were enhanced at 577 nm (Fig. 5.6).

#### Molecular weights of polyP molecules

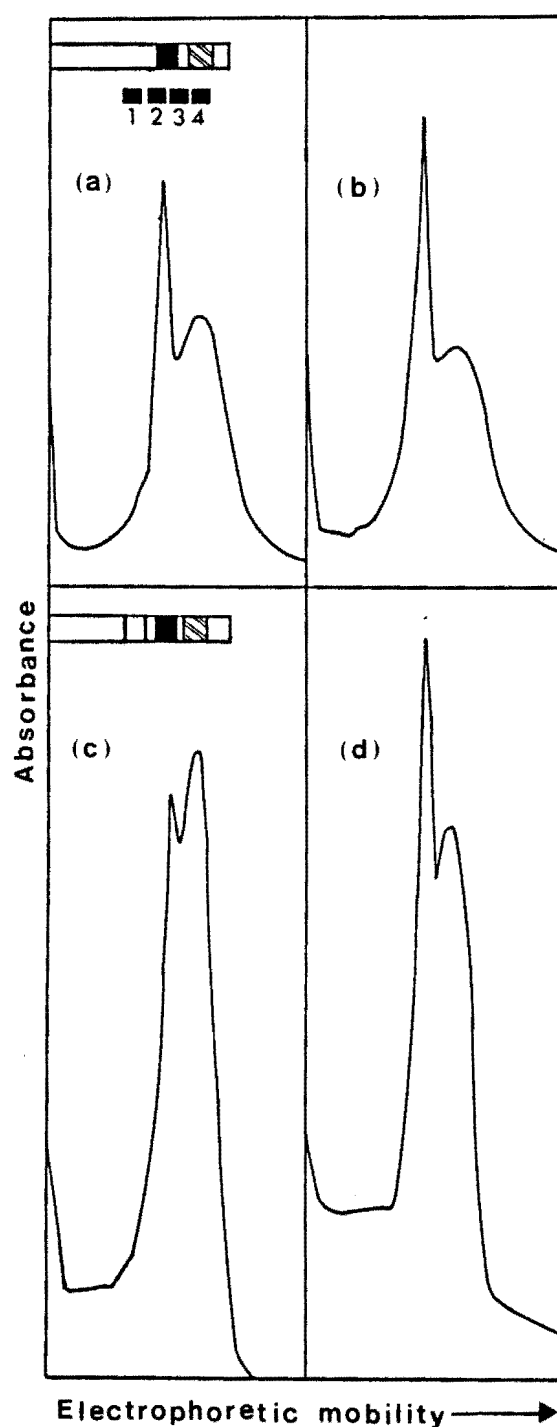
Electrophoretic bands of a range of synthetic polyP markers were run on the same gel as the mycelial extracts (Fig. 5.6). The relationship between distance of migration and



**FIG. 5.4** Frequency of metachromatic granules during growth of the endophyte of *Vaccinium macrocarpon* (●—●, granule numbers; ▲—▲, mycelial dry mass). Basal medium initially supplied with 3.23 mM sodium phytate. Vertical bars represent twice SE.



**FIG. 5.5** Absorption spectra of segments of 8.5% polyacrylamide gels stained with 0.1% toluidine blue: (a) Pink-staining metachromatic band from extracts of endophyte of *Erica hispidula*; (b) Pink-staining metachromatic band from gel on which 50  $\mu$ g synthetic polyP had been run; (c) A blue-staining nucleic acid band from extracts of endophyte of *E. hispidula*; (d) A blue-staining band from gel on which 50  $\mu$ g commercial RNA had been run; (e) A segment of gel stained only with toluidine blue; (f) Pink-staining metachromatic band from extracts of endophyte of *Rhododendron ponticum*; (g) A blue-staining nucleic-acid band from extracts of endophyte of *R. ponticum*.



**FIG. 5.6** Scans of nucleic acid-polyP phenol-detergent extracts separated on 8.5% polyacrylamide gel and stained with 0.1% toluidine blue: (a) Gel of extract of endophyte of *Rhododendron ponticum* scanned at 523 nm; (b) Same gel as in (a) scanned at 577 nm; (c) Gel of extract of endophyte of *Erica hispidula* scanned at 523 nm; (d) Same gel as in (c) scanned at 577 nm. Diagrammatic representations of the bands are shown above the scans. Solid areas are the blue, nucleic acid components; the cross-hatched area represents the pink, polyP component. Also shown are positions of synthetic polyP markers in relation to scans: (1) Type 135 (chain length 132); (2) Type 65 (chain length 65); (3) Type 45 (chain length 46); (4) Type 35 (chain length 39).

molecular weight is expressed by the following power curve equations:

$y = 27006.2x^{-0.6546}$  with  $r^2$  of 0.77 for the E. hispidula endophyte.

$y = 1357375.4x^{-1.693}$  with  $r^2$  of 0.83 for the E. mauritanica endophyte

$y = 389942.0x^{-1.435}$  with  $r^2$  of 0.98 for the R. ponticum endophyte.

The molecular weights of the polyP of the above endophytes were estimated to be  $4663 \pm 136$ ,  $4131 \pm 231$  and  $2978 \pm 288$ , respectively.

### 5.2.3 Total P and acid-labile polyP content in phenol-detergent extracts

The total P and acid-labile polyP content of the endophytes of E. hispidula and R. ponticum were similar (the latter being 11 and 8%, respectively of total P) (Table 5.1). The endophyte of E. mauritanica showed a higher acid-labile polyP content but this formed a smaller proportion (7%) of a higher total P content. The similarity between the acid-labile polyP content of the extracts and the total P of the pink, metachromatic gel bands confirmed the presence of a predominantly acid-labile fraction. Root systems of seedlings of V. macrocarpon were inoculated with the

**TABLE 5.1** Acid-labile polyP content of phenol-detergent extracts and total P of isolated endophytes grown in culture on basal medium and 3.23 mM sodium phytate for 8d and aseptically infected root systems of Vaccinium macrocarpon

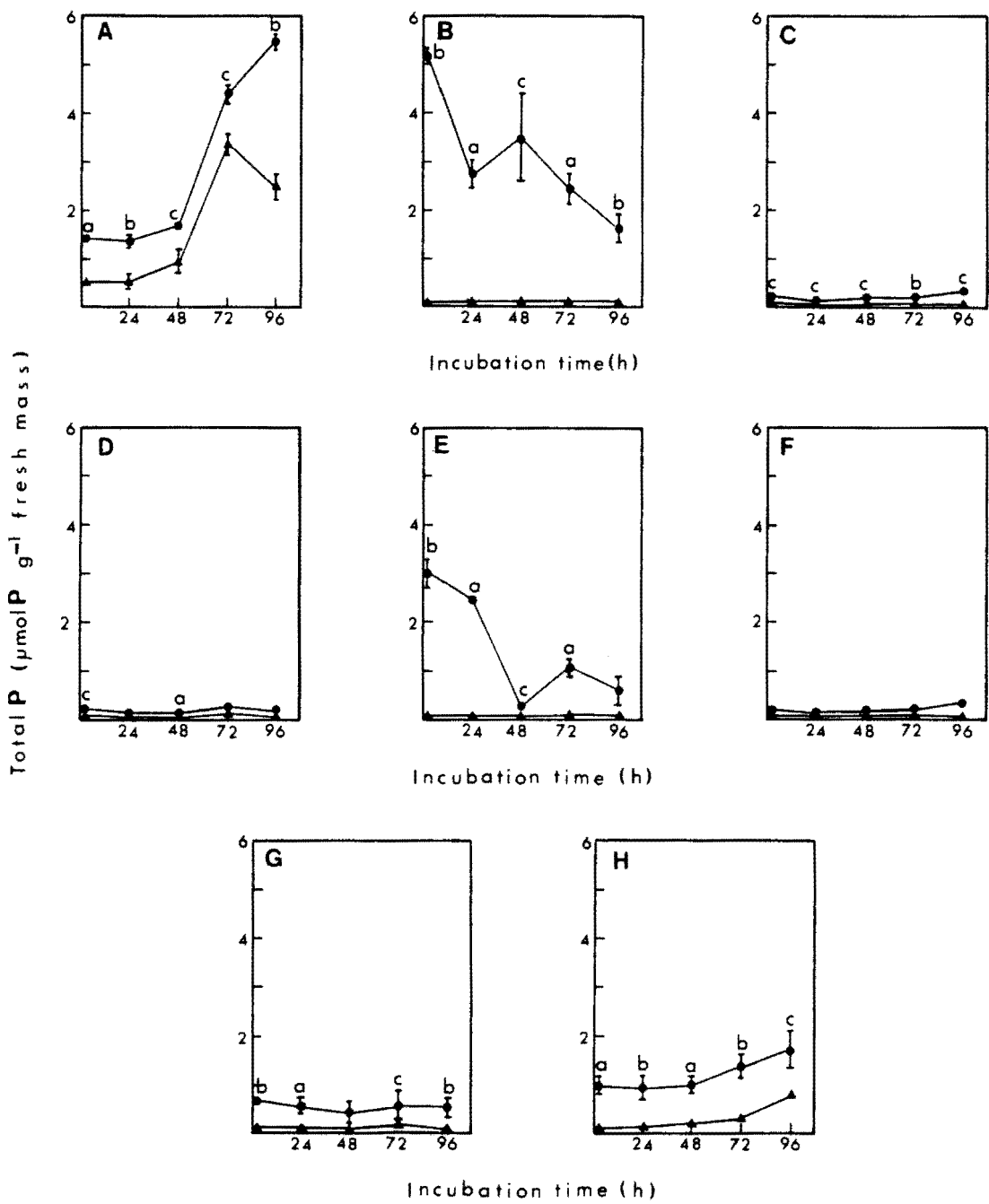
	$\mu\text{mol g}^{-1}$ fresh mass $\pm$ SE			(%) of total
	Acid-labile P	Total P of pink bands	Total P	Acid-labile P
Endophyte				
<u>E. hispidula</u>	1.9+0.1	1.7+0.3	17.5+2.0	10.9
<u>R. ponticum</u>	1.4+0.3	1.1+0.2	18.2+2.1	7.7
<u>E. mauritanica</u>	2.2+0.2	2.1+0.5	33.6+1.4	6.6
Root systems				
Infected with				
<u>E. hispidula</u> endophyte	1.0+0.2 <sup>a</sup>	-	9.5+2.0	10.5
Infected with				
<u>E. mauritanica</u> endophyte	3.9+0.3 <sup>b</sup>	-	19.8+3.2 <sup>a</sup>	19.7
Uninfected	0.3+0.1	-	10.4+2.6	3.0

a and b are significantly different from uninfected root at 0.05 and 0.001 levels respectively; - signifies not determined.

endophytes of E. hispidula and E. mauritanica and after three months incubation, the proportions of acid-labile polyP of total P were 10 and 20%, respectively. Infection with E. mauritanica endophyte also resulted in a two-fold increase in the total P of the root system when compared with uninfected roots, which had a low acid-labile P content of 3% of the total P. Infection with the endophyte of E. hispidula did not result in a similar increase in the total P of the root system.

#### 5.2.4 The effect of P starvation on the endogenous P status of isolated endophytes of E. hispidula grown on high and low levels of orthophosphate

When mycelia grown on high and low levels of  $\text{KH}_2\text{PO}_4$  were starved of P for four days and total P levels in fractions assessed, the levels of TCA-soluble orthophosphate rose rapidly from day one in both sets of mycelia although in the low P-fed mycelia this fraction declined after three days (Fig. 5.7). In contrast, negligible amounts of P in the form of TCA-insoluble orthophosphate were present in either the high or low P-fed mycelia. A high amount of TCA-soluble polyP was present in the high P-fed mycelia at day zero but then declined. TCA-soluble polyP was negligible in the low P-fed mycelia. A similar trend was found in the TCA-insoluble polyP fraction although with a lower content in the high P-fed mycelia. The TCA-soluble non-labile P fractions showed low levels but with significant differences



**FIG. 5.7** Total P in fractions of 8-d-old mycelia of endophyte of *Erica hispidula* grown on basal medium containing either 3.23 mM or 0.16 mM KH<sub>2</sub>PO<sub>4</sub>, then incubated for 4d in fresh basal medium lacking P (●—●, 3.23 mM; ▲—▲, 0.16 mM). A, TCA-soluble orthophosphate; B, TCA-soluble polyP; C, TCA-soluble non-labile polyP; D, TCA-insoluble orthophosphate; E, TCA-insoluble polyP; F, TCA-insoluble non-labile P; G, Residue P; H, lipid P. Vertical bars represent twice SE. a, b and c represent significant differences at 0.001, 0.01 and 0.05 levels respectively between starved high P-fed mycelia and starved low P-fed mycelia. Results are the means of 4 replicates.



between high and low P-fed mycelia. Levels of the TCA-insoluble non-labile P fractions were also low. Levels of residue P were low with little variation over time. Significantly different amounts of lipid P in high and low P-fed mycelia were present at day zero but there appeared a similar pattern of accumulation with time

#### 5.2.5 Activated charcoal adsorption of possible contaminants of BaCl<sub>2</sub> precipitates

The addition of activated charcoal to the TCA-soluble and insoluble filtrates of homogenates prior to precipitation by BaCl<sub>2</sub> led to a significant increase in TCA-soluble non-labile P fractions when compared with untreated samples (Table 5.2). No significant differences were found between the levels of labile polyP fractions of treated and untreated samples which suggests little contamination of BaCl<sub>2</sub> precipitates by other labile P compounds.

#### 5.2.6 Phytic acid content of mycelia of the endophyte of E. hispidula

Phytic acid in seven-day-old mycelia grown on basal medium containing 3.23 mM KH<sub>2</sub>PO<sub>4</sub> was determined as  $1.26 \pm 0.21$   $\mu\text{mol P g}^{-1}$  fresh mass which represented 6.2% of the total P.

TABLE 5.2 The effect of the addition of activated charcoal to TCA-soluble and -insoluble extracts on P fraction levels of mycelia of the endophyte of Erica hispidula grown for 7d on basal medium containing 3.23 mM  $\text{KH}_2\text{PO}_4$

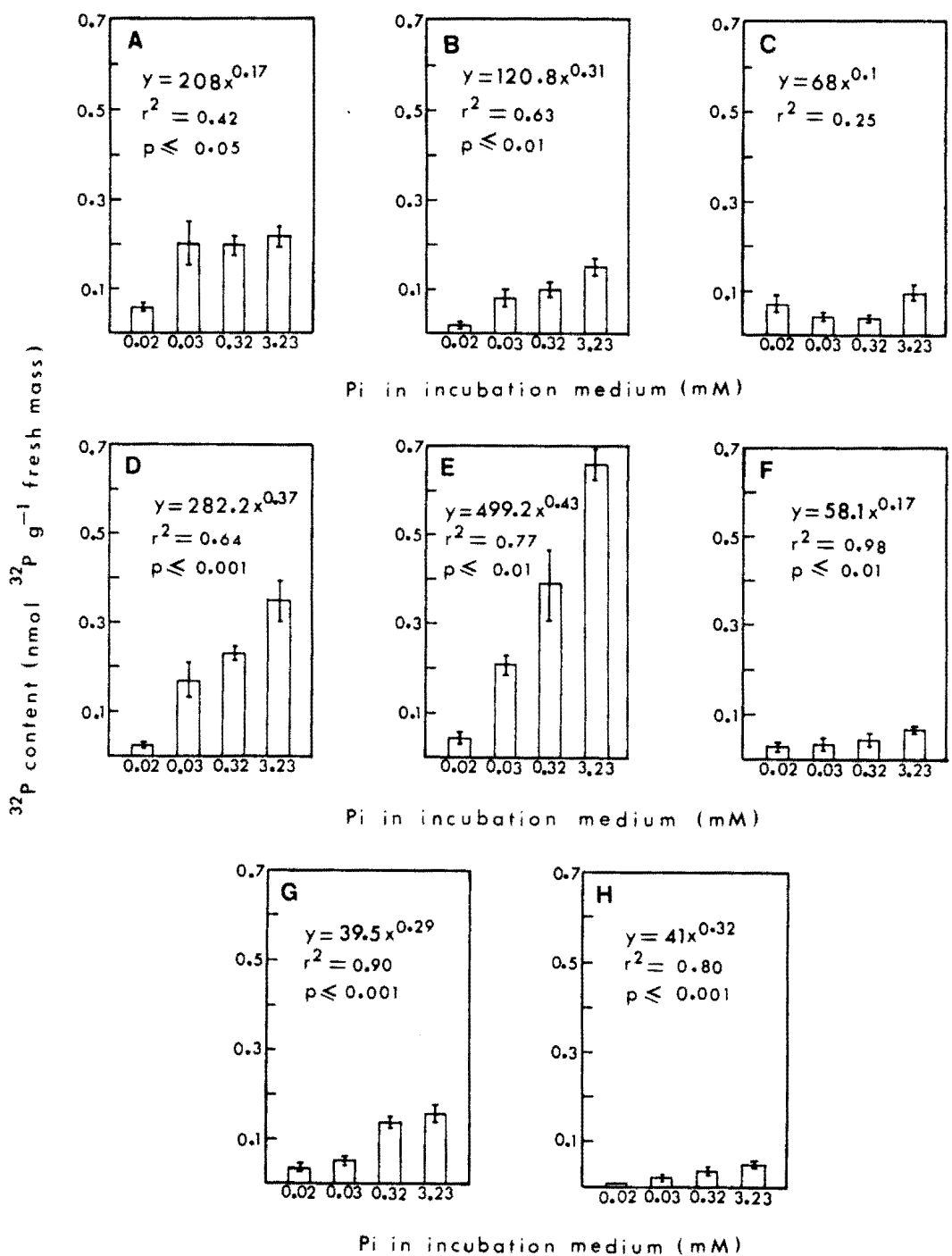
<u>P Fraction</u>	<u>Total P (<math>\mu\text{mol P g}^{-1}</math> fresh mass +SE)</u>	
	Charcoal adsorption	No charcoal adsorption
TCA-soluble orthophosphate	3.5 $\pm$ 1.2	1.9 $\pm$ 0.4
TCA-insoluble orthophosphate	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1
TCA-soluble polyP	1.8 $\pm$ 0.5	1.2 $\pm$ 0.04
TCA-soluble non-labile P	0.4 $\pm$ 0.04	0.2 $\pm$ 0.05 <sup>a</sup>
TCA-insoluble polyP	2.9 $\pm$ 1.0	4.5 $\pm$ 0.3
TCA-insoluble non-labile P	0.5 $\pm$ 0.1	0.5 $\pm$ 0.04
Residue P	10.6 $\pm$ 2.4	10.1 $\pm$ 1.2

a represents significant difference at 0.05 level

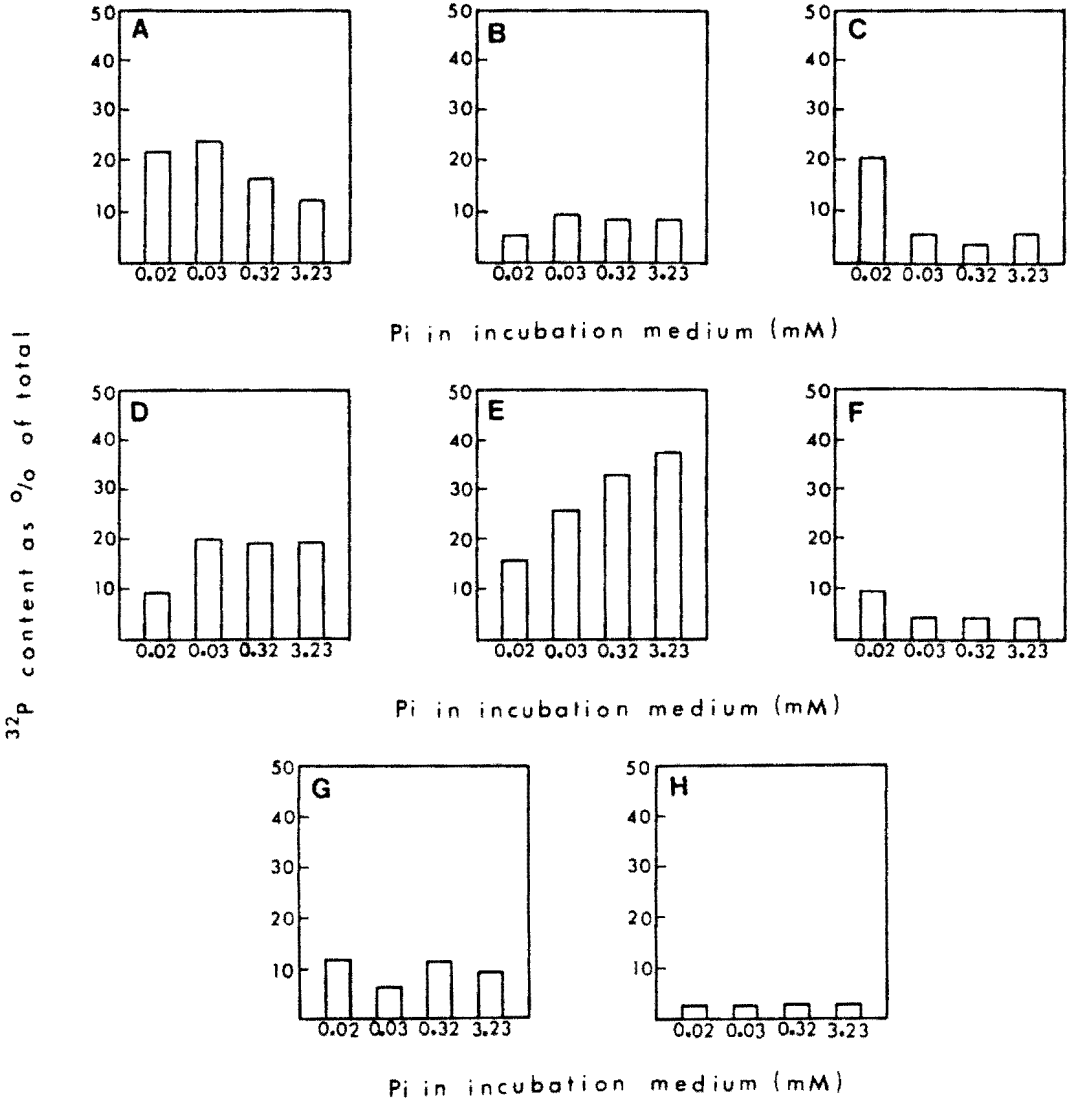
### 5.2.7 Fractionation of $^{32}\text{P}$ in mycelia of the endophyte of *E. hispidula* after incubation in $\text{KH}_2^{32}\text{PO}_4$ and various concentrations of P

The potential of polyP as a sink for excess P absorbed was assessed by the use of  $^{32}\text{P}$  as a tracer when mycelia were incubated for 24 hours in different concentrations of external P. When specific concentrations of  $^{32}\text{P}$  were diluted with concentrations of  $\text{KH}_2\text{PO}_4$ , the relationship between  $^{32}\text{P}$  incorporated by mycelia into different fractions and the levels of external unlabelled P took the form of power curves in the TCA-soluble polyP, TCA-insoluble orthophosphate, TCA-insoluble polyP, TCA-insoluble non-labile P, residue P and lipid P fractions (Fig. 5.8). The TCA-insoluble polyP fraction showed the highest levels of P incorporated with lower levels accumulated as TCA-soluble polyP and substantial amounts accumulated in the TCA-soluble and -insoluble orthophosphate fractions. When  $^{32}\text{P}$  levels in each fraction were expressed as a percentage of the total P supplied, only the TCA-insoluble polyP fraction showed an increase in  $^{32}\text{P}$  levels with external unlabelled  $\text{KH}_2\text{PO}_4$  (Fig. 5.9).

Mycelia of the endophyte of *E. hispidula* were also grown for eight days in the basal medium containing 3.23 mM glucose-6-phosphate and fractionated after incubation for two hours in glucose-6- $^{32}\text{P}$ . Six percent of the absorbed  $^{32}\text{P}$  accumulated as total acid-labile polyP.



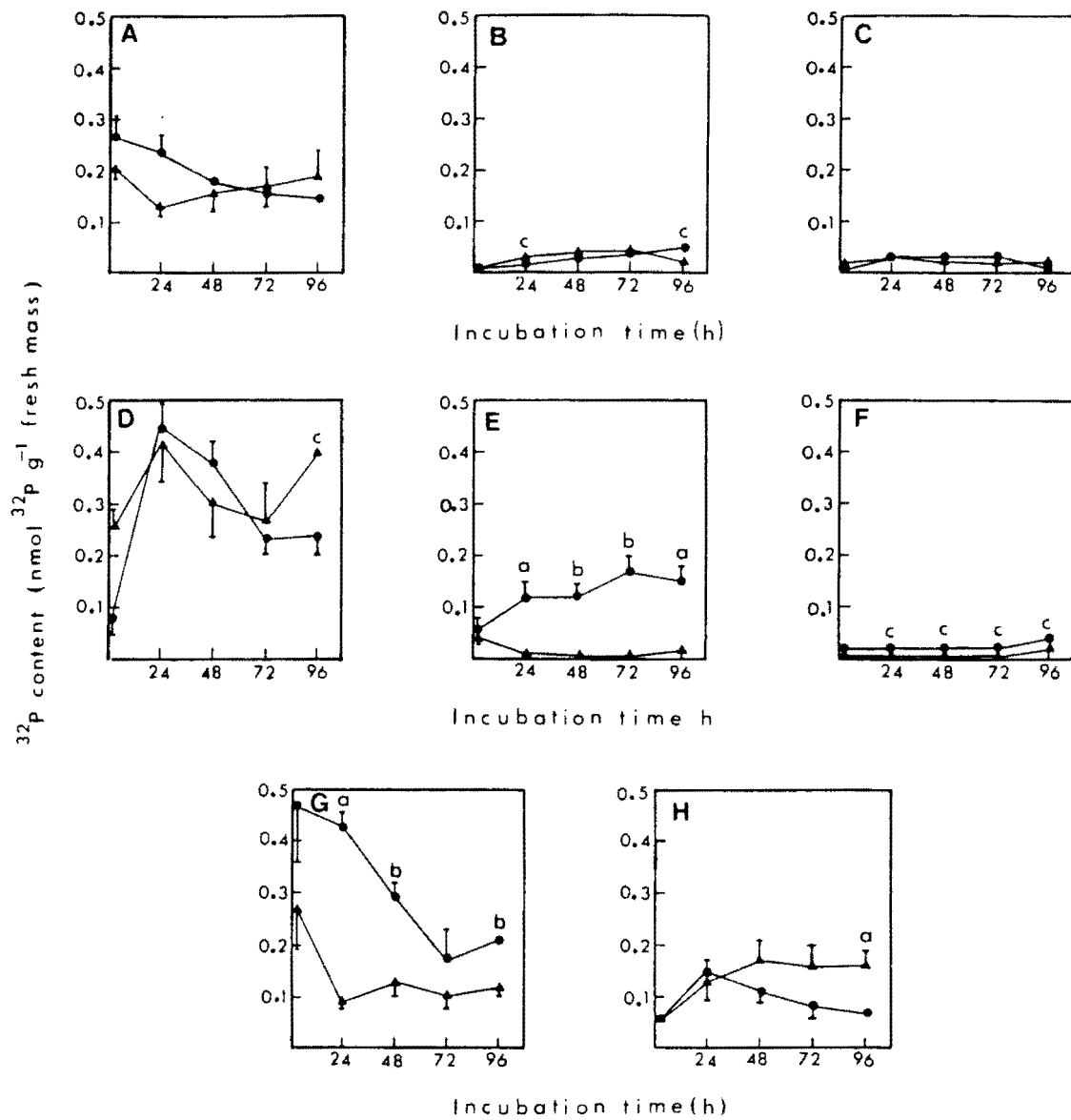
**FIG. 5.8** Levels of  $^{32}\text{P}$  in P fractions of mycelia of endophyte of *Erica hispidula* grown for 7 d on basal medium with 3.23 mM  $\text{KH}_2\text{PO}_4$  and incubated for 24 h in phthalate buffer pH 5.5 with 40  $\mu\text{Ci dm}^{-3}$   $\text{KH}_2^{32}\text{PO}_4$  and various concentrations  $\text{KH}_2\text{PO}_4$ . A, TCA-soluble orthophosphate; B, TCA-soluble polyP; C, TCA-soluble non-labile P; D, TCA-insoluble orthophosphate; E, TCA-insoluble polyP; F, TCA-insoluble non-labile P; G, residue P; H, lipid P. Power curve equations inset. Vertical bars represent twice SE. Analysis of variance significance levels shown. Results are the means of 4 replicates.



**FIG. 5.9** Percentage of  $^{32}\text{P}$  incorporated into P fractions of mycelia of endophyte of *Erica hispidula* grown for 7 d on basal medium containing 3.23 mM  $\text{KH}_2\text{PO}_4$  and incubated for 24 h in phthalate buffer pH<sup>2</sup><sub>4</sub> 5.5 with 40  $\mu\text{Ci dm}^{-3}$   $\text{KH}_2^{32}\text{PO}_4$  and various concentrations  $\text{KH}_2\text{PO}_4$ . A, TCA-soluble orthophosphate; B, TCA-soluble polyP; C, TCA-soluble non-labile P; D, TCA-insoluble orthophosphate; E, TCA-insoluble polyP; F, TCA-insoluble non-labile P; G, residue P; H, lipid P. The results are the means of 4 replicates.

5.2.8 The fractionation of  $^{32}\text{P}$  over time in mycelia of the endophyte of *E. hispidula* grown on high and low levels of orthophosphate

The dynamics of absorption and exchange within the organism at a particular growth stage can be better assessed if monitored over time. The function of polyP as a sink for P must also be seen in relation to the endogenous P status of the organism. Mycelia grown at a high (3.23 mM) and a low (0.16 mM) concentration of orthophosphate and fed  $\text{KH}_2^{32}\text{PO}_4$  were fractionated over a four-day period. In both high and low P-fed mycelia, there was a substantial accumulation of  $^{32}\text{P}$  as TCA-soluble and -insoluble orthophosphate (Fig. 5.10).  $^{32}\text{P}$  levels in the TCA-insoluble orthophosphate fraction rose rapidly during 24 hours in both high and low P-fed mycelia but declined over the following three days. P levels in the TCA-soluble polyP fraction were low in both high and low P-fed mycelia. Levels of  $^{32}\text{P}$  in the TCA-insoluble polyP fraction gradually increased with time in high P-fed mycelia but remained significantly lower in low P-fed mycelia. The TCA-insoluble non-labile P fraction showed low but significantly different amounts of  $^{32}\text{P}$  in high and low P-fed mycelia. Within two hours, there had been a substantial accumulation of  $^{32}\text{P}$  in the residue P fraction in the high P-fed mycelia followed by a marked decline over four days. The same trend was apparent in the low P-fed mycelia although levels were significantly lower.  $^{32}\text{P}$  in the lipid fraction accumulated to the same level in



**FIG. 5.10**  $^{32}\text{P}$  in fractions of 8-d-old mycelia of endophyte of *Erica hispidula* grown on basal medium containing either 3.23 mM or 0.16 mM  $\text{KH}_2\text{PO}_4$  and then incubated for 4d in fresh basal medium<sup>2</sup> with 100  $\mu\text{Ci dm}^{-3}$   $\text{KH}_2\text{PO}_4$  (●—●, 3.23 mM; ▲—▲, 0.16 mM). A, TCA-soluble orthophosphate; B, TCA-soluble polyP; C, TCA-soluble non-labile P; D, TCA-insoluble orthophosphate; E, TCA-insoluble polyP; F, TCA-insoluble non-labile P; G, residue P; H, lipid P. Vertical bars represent once SE. a, b and c represent significant differences at 0.001, 0.01 and 0.05 levels respectively between high P-fed and low P-fed mycelia. Results are the mean of 4 replicates.

high and low P-fed mycelia in 24 hours and thereafter remained either constant (low P) or declined (high P).

### 5.3 DISCUSSION

The presence of polyP in pure cultures of the endophyte and mycorrhizal root systems of ericaceous seedlings has been confirmed. The metachromatic granules resemble closely those of ectomycorrhizas of eucalypts and pines (Ashford et al., 1975; Ling-Lee et al., 1975; Chilvers and Harley, 1980) and vesicular-arbuscular mycorrhizas (Cox et al., 1975). The range of granule sizes were similar to those of beech mycorrhizas incubated in 1 mM orthophosphate and the sensitivity of granule numbers to the concentration of the external P source is similar to the pattern observed in beech mycorrhizas and an ectomycorrhizal fungus in pure culture (Chilvers and Harley, 1980; Lapeyrie et al., 1984). The decline in granule numbers after an organic P concentration of 0.32 mM may be due to the end-product inhibition of the phosphatase enzymes (Pearson and Read, 1975) which would have reduced the amount of P available for uptake. The accumulation of granules in hyphae in media without the addition of an external P source was most likely due to the presence of residual P in the yeast extract of the basal medium. The polyP granules accumulated during the lag phase of growth. In Corynebacterium xerosis (Neiss. et Kusch.) Bergey, numbers of granules increased during the lag



phase but declined during the exponential phase (Hughes and Muhammed, 1962).

The phenol-detergent extraction technique of Callow et al. (1978) was used to isolate polyP and nucleic acid in a relatively undegraded form and the polyP was finally characterized by means of polyacrylamide gel electrophoresis. Callow et al. (1978) were able to determine the molecular weight and chain length of the polyP in VA mycorrhizas as polyphosphates have a constant charge to mass ratio and their mobility in the gels is a function of their molecular size. In this study, the molecular weights of 3 000 to 4 700 from ericoid endophytes were considerably lower than the figure of 20 800 for the polyP of vesicular-arbuscular mycorrhizal fungi (Callow et al., 1978) although the possibility of chain degradation during extraction should not be discounted. The proportion of acid-labile polyP to total P in the phenol-detergent extract of the cultured endophyte was 7 to 11% which is in the same range estimated by Capaccio and Callow (1982) in phenol detergent extracts of isolated internal vesicular-arbuscular endophytes. The polyP content of ericoid mycorrhizal root systems was significantly greater than that of the non-mycorrhizal controls indicating the polyP to be largely fungal in origin. The higher proportion of polyP in the root systems inoculated with the endophyte from E. mauritanica could have been due to a heavier mycorrhizal infection observed. However, as the higher total P content

of these infected seedlings was correlated with a high concentration of P in the isolated endophyte, the mycorrhizal fungus of E. mauritanica may also be able to accumulate more phosphorus.

When high P-fed mycelia of the endophyte of E. hispidula were starved of P, the initially high levels of TCA-soluble and -insoluble polyP fell rapidly with a concomitant rise in TCA-soluble orthophosphate. Low P-fed mycelia contained negligible polyP and  $^{32}\text{P}$  orthophosphate, when supplied, was not transformed into polyP. These trends confirm the potential of the molecule to be a form of P storage when P is in surplus and to release P into the orthophosphate fraction under conditions of P deprivation. Mycelia showed an accumulation and transformation of  $^{32}\text{P}$  into various fractions which was enhanced by external P supply. Only the TCA-insoluble polyP (long-chain polyP) fraction showed increased levels of  $^{32}\text{P}$  as a proportion of the total P (Figs. 5.8 and 5.9). This trend suggests a very rapid incorporation of P into TCA-insoluble polyP at the expense of the other fractions. Evidence by Juni et al. (1947), Wiame (1949), Harold (1962) and Harold (1966) indicated that acid-insoluble polyP is a more active form with a higher turnover rate than acid-soluble polyP. However, the rates of turnover of the polyP pools may be the same but they stand in a 'precursor-product' relationship with the longer-chain molecules being formed first (Harold, 1966). When high P-fed

mycelia were fed  $^{32}\text{P}$  alone, accumulation occurred rapidly in the residue P fractions (Fig. 5.10) which has been shown to contain nucleic acids and phosphoproteins (Schmidt and Thannhauser, 1945). This fraction then became depleted in favour of TCA-insoluble orthophosphate and TCA-insoluble polyP synthesis. In many microorganisms, the interruption of nucleic acid synthesis (usually by the absence or depletion of an essential metabolite) is directly linked to a concomitant rise in polyP synthesis and vice versa (Harold, 1962; Harold and Sylvan, 1963; Harold, 1966). It is unlikely that essential nutrients became exhausted from fresh basal media after only 24 hours and to suggest reasons for the cessation of  $^{32}\text{P}$  accumulation in the residue fractions would be speculative in the absence of further empirical evidence.

High levels of acid-soluble polyP (67% of total P prior to starvation period) were found in fractions in comparison with levels determined in the phenol-detergent extracts. The difference in P source (orthophosphate as opposed to sodium phytate) may have accounted for part of the discrepancy since the endophyte does not grow equally well on both sources (Fig. 3.1). However, it is more likely that the difference in the extraction techniques was the primary factor contributing to discrepancies in polyP levels. Levels of  $^{32}\text{P}$  incorporated into acid-labile fractions (23 to 46% of total  $^{32}\text{P}$ ) are in the same range as those of excised

beech mycorrhizas (Harley and McCready, 1981). Martin et al. (1983) and Rolin et al. (1984) found low levels of polyP (9 to 17%) in the acid-soluble barium acetate precipitate of ectomycorrhizal fungi but none in the acid-insoluble precipitate and polyP levels were found to be unrelated to external P levels. The extraction procedure used by these workers included determinations of RNA and DNA levels and specific identification of polyP by  $^{31}\text{P}$  NMR (nuclear magnetic resonance) and thin-layer chromatography. Acid-labile nucleotides, nucleic acids or sugar phosphates are able to contaminate TCA extracts and nucleic acids may form complexes with the acid-insoluble polyP precipitate (Harold, 1966). Although the fractionation experiments did not include adsorption of contaminants by activated charcoal prior to barium chloride precipitation, a significant difference was only found in the TCA-soluble non-labile P fraction when control extractions were made, and thus the data remained uncorrected. Despite the acid-labile test for polyP fractions, the extraction and fractionation procedure of Aitchison and Butt (1973) is not equivocal without chromatography and metachromasy (Bieleski, 1973) and polyP estimates in these fractions should not be regarded as conclusive. Nevertheless, biochemical and histochemical evidence emphasizes the potential importance of polyP in the overall P metabolism of ericoid endophytes. The endophyte also shows an ability to utilise glucose-6-phosphate and

be an important inhibiting factor. In the fynbos vegetation, there is a seasonal pattern in nitrogen mineralization (Stock et al., Stock and Lewis, in press) and total phosphorus levels in the Clovelly soil (Mitchell et al., 1984). Decomposition of the leaf litter does not show a seasonal pattern but its rate is slow (Mitchell et al., in press). The slow decomposition rates are attributed to the poor quality of litter which is due to its high lignin content and C : N ratios (Mitchell et al., in press).

Although the phosphorus contents of the acidic sandy soils are low, the bulk of it is organically bound (27-60%) and thus acid phosphatases secreted by the microorganisms would be important in the catalytic release of orthophosphate from bound complexes. In this study, the endophyte was isolated from the fine hair roots of E. hispidula and E. mauritanica and grown in culture. The former was shown to possess active fractions of acid phosphatase, particularly the enzymes either associated with the cell surface or released into the external medium (Table 3.1). The optimum pH of phosphatase activity (i.e. pH 2.0 - 6.0) was within the range for the fynbos sandy soil (Mitchell et al., 1984), and the enzymes showed a wide substrate specificity (Table 3.3). Under field conditions, the endophyte would be able to achieve maximal access to organic P substrates during the periodic cycles of favourable environmental conditions. The extramatrical hyphae acting as physical extensions of the

al., 1983). On these strongly-leached, acidic soils, winter- spring is probably the main period of nutrient release from decomposing litter, but this has not been substantiated. Read (1978) has noted that in the heathlands of the Cape, mycorrhizal infection of Erica spp. is most active during the spring but negligible during the late summer months at a time when many species of Erica spp. flower and set seed (Pierce, 1984). Duddridge and Read (1982b) have shown that the longevity of the mycorrhizal association in cells of Rhododendron ponticum only lasted approximately seven weeks. If this estimate is true for Erica spp. in the fynbos, then the advantages gained from maximal mineralization and absorption of phosphates will be realised through rapid translocation of P to the intracellular hyphae and then the storage of excess P for release to the host plant during the period of both shoot extension and flower production.

The potential role of polyP as a storage molecule in ericoid mycorrhizas was confirmed in this study. There were substantial differences in the levels of total polyP between mycelia of the endophyte of E. hispidula grown on a high P and a low P medium (Fig. 5.7). In high P-fed mycelia polyP levels declined during P starvation with a concomitant rise in the levels of orthophosphate. If 8-day-old mycelia were fed increasing concentrations of P, labelled with  $^{32}\text{P}$  orthophosphate, the acid-insoluble polyP fraction showed the

best concomitant increase in P levels (Figs. 5.8 and 5.9). Mycorrhizal seedlings contained significantly higher levels of polyP than non-mycorrhizal seedlings (Table 5.1). These results suggest the operation of a polyP cycle (Fig. 1.1) whereby absorbed P is initially incorporated into long-chain acid-insoluble polyP which is broken down to shorter chains of acid-soluble polyP (Harold, 1966). Finally, orthophosphate is produced for transfer across the hyphae/host interface. During the late summer and autumn of the south western Cape, when mycorrhizal infections of the root system of Erica spp. diminish and the carbohydrate drain on the host is reduced, nutrients are probably being directed to the flowers either from the root system or internally cycled from the leaves prior to leaf fall.

As well as the South African endophytes, polyP was identified in the European endophytes with granules accumulating in the exponential phase of growth and in response to the concentration of P in the external medium (Figs. 5.3 and 5.4). However, there appear to be fundamental differences in the P nutrition between South African and European endophytes. The South African endophyte of E. hispidula: (1) possessed higher activities of acid phosphatases (especially the extracellular fraction), (2) took a shorter time to reach the stationary phase of growth in culture, (3) showed a different ability to use phytates as a P source and (4) has shown a greater ability to use

inositol as a carbon source (Mitchell and Read, 1985). These differences may be of an adaptive nature, related to differences in moisture regimes, soil organic matter and nutrient levels between northern and southern hemisphere heathlands and further research will establish their validity. The apparent low phytase ability of the acid phosphatases of the ericoid endophyte and the absence of an alkaline phosphatase suggests that the enzyme physiology of ericoid mycorrhizas may not be the same as that of either ectomycorrhizas or VA mycorrhizas. Studies on the enzymes involved in polyphosphate metabolism have been undertaken on VA mycorrhizas (Capaccio and Callow, 1982) but research in this field on ericoid mycorrhizas needs to be pursued. The studies of Read and Stribley (1973), Stribley and Read (1974b, 1976, 1980) indicate the ability of ericoid mycorrhizas to utilise organic sources of nitrogen which the host cannot directly use and underlines the importance of the mycorrhizal association in the nitrogen nutrition of ericaceous plants. This study has concentrated on P utilization by ericoid endophytes in culture but confirms their importance in the P nutrition of Erica spp. This will have to be studied further by synthesising mycorrhizal root systems of the original host plant which will enable the distribution patterns of P within the host plant to be investigated.



# REFERENCES

- Aitchison, R.A. & Butt, V.S. (1973). The relation between the synthesis of inorganic polyphosphate and phosphate uptake by Chlorella vulgaris. Journal of Experimental Botany, 24 (80), 497-510.
- Alexander, I.J. & Hardy, K. (1981). Surface phosphatase activity of Sitka spruce mycorrhizas from a serpentine site. Soil Biology and Biochemistry, 13, 301-305.
- Allen, S.E., Grimshaw, H.M. Parkinson, J.A. & Quarmby, G. (1974). Chemical Analysis of Ecological Materials. Blackwell Scientific Publications, Oxford.
- Anderson, G. (1967). Nucleic acid derivatives and organic phosphates. In Soil Biochemistry (Ed. by A.D. McLaren & G.H. Peterson), pp. 67-89. Marcel Dekker, Inc., N.Y.
- Anderson, G., Williams, E.G. & Moir, J. (1974). A comparison of the sorption of inorganic orthophosphate and inositol hexaphosphate by six acid soils. Journal of Soil Science, 25, 51-62.
- Appiah, M.R. & Thomas, R.L. (1982). Inositol phosphate and organic phosphorus contents and phosphatase activity of some Canadian and Ghanaian soils. Canadian Journal of Soil Science, 62, 31-38.
- Arnold, W.N. & Garrison, R.G. (1979). An  $\text{Fe}^{3+}$ -activated acid phosphatase in Saccharomyces rouxii. Journal of Biological Chemistry, 254 (11), 4919-4925.
- Ashford, A.E., Ling-Lee, M. & Chilvers, G.A. (1975). Polyphosphate in Eucalypt mycorrhizas : a cytochemical demonstration. The New Phytologist, 74, 447-453.
- Bartlett, E.M. & Lewis, D.H. (1973). Surface phosphatase activity of mycorrhizal roots of beech. Soil Biology and Biochemistry, 5, 249-257.
- Basha, Sheikh Mehboob. (1984). Purification and characterization of an acid phosphatase from peanut (Arachis hypogaea) seed. Canadian Journal of Botany, 62, 385-391.
- Beever, R.E. & Burns, D.J.W. (1977). Adaptive changes in phosphate uptake by the fungus Neurospora crassa in response to phosphate supply. Journal of Bacteriology, 132(2), 520-525.

- Beever, R.E. & Burns, D.J.W. (1980). Phosphorus uptake, storage and utilization by fungi. In Advances in Botanical Research, vol. 8 (Ed. by H.W. Woolhouse), pp. 127-219. Academic Press, London.
- Bennett, J. & Scott, K.J. (1971). Inorganic polyphosphates in the wheat stem rust fungus and in rust-infected wheat leaves. Physiological Plant Pathology, 1, 185-198.
- Bieleski, R.L. (1973). Phosphate pools, phosphate transport and phosphate availability. Annual Review of Plant Physiology, 24, 225-252.
- Bojović-Cvetić, D. & Vujičić, R. (1982). Acid phosphatase localisation and distribution in Aspergillus flavus. Transactions of the British Mycological Society, 79(1), 137-141.
- Bonfante-Fasola, P. & Gianinazzi-Pearson, V. (1979). Ultrastructural aspects of endomycorrhiza in the Ericaceae. I. Naturally infected hair roots of Calluna vulgaris L. Hull. The New Phytologist, 83, 739-744.
- Bonfante-Fasola, P., Berta, G. & Gianinazzi-Pearson, V. (1981). Ultrastructural aspects of endomycorrhizas in the Ericaceae. II. Host-endophyte relationships in Vaccinium myrtillus L. The New Phytologist, 89, 219-224.
- Bonfante-Fasola, P. & Gianinazzi-Pearson, V. (1982). Ultrastructural aspects of endomycorrhiza in the Ericaceae. III. Morphology of the dissociated symbionts and modifications occurring during their reassociation in axenic culture. The New Phytologist, 91, 691-704.
- Borst-Pauwels, G.W.F.H. (1976). Discrimination criteria for apparent two-site transport models. Journal of Theoretical Biology, 56, 191-204.
- Bowen, G.D., Bevege, G.I. & Mosse, B. (1975). Phosphate physiology of vesicular-arbuscular mycorrhizae. In Endomycorrhizas (Ed. by F.E. Sanders, B. Mosse & P.B. Tinker), pp. 241-260. Academic Press, London and New York.
- Burns, D.J.W. & Beever, R.E. (1977). Kinetic characterization of the two phosphate uptake systems in the fungus Neurospora crassa. Journal of Bacteriology, 132(2), 511-519.

- Burns, D.J.W. & Tucker, S.A. (1977). An evaluation of fitting methods for the sum of two hyperbolas : application to uptake studies. European Journal of Biochemistry, 81, 45-52.
- Calleja, M., Mousain, D., Lecouvreur, B. and d'Auzac, J. (1980). Influence de la carence phosphatée sur les activités phosphatases acides de trois champignons mycorrhiziens. Hebeloma edurum Metrod., Suillus granulatus (L. ex FR.) O. Kuntze et Pisolithus tinctorius (Pers.) Coker et Couch. Physiologie Vegetale 18(3), 489-504.
- Calleja, M. & d'Auzac, J. (1983). Activités phosphatases et carence phosphatée chez des champignons superieurs. Canadian Journal of Botany, 61, 79-86.
- Callow, J.A., Capaccio, L.C.M., Parish, G. & Tinker, P.B. (1978). Detection and estimation of polyphosphate in vesicular-arbuscular mycorrhizas. The New Phytologist, 80, 125-134.
- Capaccio, L.C.M. & Callow, J.A. (1982). The enzymes of polyphosphate metabolism in vesicular-arbuscular mycorrhizas. The New Phytologist, 91, 81-91.
- Cheshire, M.V. & Anderson, G. (1975). Soil polysaccharides and carbohydrate phosphates. Soil Science, 119, 356-362.
- Chilvers, G.A. & Harley, J.L. (1980). Visualization of phosphate accumulation in beech mycorrhizas. The New Phytologist, 84, 319-326.
- Chilvers, G.A., Lapeyrie, F.F. & Douglass, P.A. (1985). A contrast between Oomycetes and other taxa of mycelial fungi in regard to metachromatic granule formation. The New Phytologist, 99, 203-210.
- Cooper, K.M. & Tinker, P.B. (1981). Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. IV. Effect of environmental variables on movement of phosphorus. The New Phytologist, 88, 327-339.
- Cornish-Bowden, A. & Eisenthal, R. (1974). Statistical considerations in the estimation of enzyme kinetic parameters by the direct linear plot and other methods. Biochemical Journal, 139, 721-730.
- Cosgrave, D.J. (1967). Metabolism of organic phosphates in soil. In Soil Biochemistry (Ed. by A.D. McLaren & G.H. Peterson), pp. 216-228. Marcel Dekker, Inc., N.Y.

- Cosgrave, D.J. (1980). Inositol phosphates : their Chemistry, Biochemistry and Physiology. Vol. 4. Studies in Organic Chemistry. Elsevier Scientific Publishing Company, Amsterdam.
- Cox, G. & Tinker, P.B. (1976). Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. I. The arbuscle and phosphorus transfer : a quantative ultrastructural study. The New Phytologist, 77, 371-378.
- Cox, G., Moran, K.J., Sanders, F., Nockolds, G. & Tinker, P.B. (1980). Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. III. Polyphosphate granules and phosphorus translocation. The New Phytologist, 84, 649-659.
- Crane, R.K. (1958). Use of charcoal to separate mixtures of inorganic, ester and nucleotide phosphates. Science, 127, 285-286.
- Cress, W.A., Throneberry, G.O. & Lindsay, D.L. (1979). Kinetics of phosphorus absorption by mycorrhizal and non-mycorrhizal tomato roots. Plant Physiology, 64, 484-487.
- Davis, B.J. (1964). Disc electrophoresis II : Method and application to human serum proteins. Annals of New York Academy of Science, 121, 404-427.
- Duclos, J.L. & Fortin, J.A. (1983). Effect of glucose and active charcoal on in vitro synthesis of ericoid mycorrhizas with Vaccinium spp. The New Phytologist, 94, 95-102.
- Duddridge, J.A. and Read, D.J. (1982a). An ultrastructural analysis of the development of mycorrhizas in Monotropa hypopitys. L. The New Phytologist, 92, 203-214.
- Duddridge, J. and Read, D.J. (1982b). An ultrastructural analysis of the development of mycorrhizas in Rhododendron ponticum. Canadian Journal of Botany, 60, 2345-2356.
- Ebel, J.P., Colas, J. & Muller, S. (1958a). Recherches cytochimiques sur les polyphosphates inorganiques contenus dans les organismes vivants. II. Mise au point de méthodes de detection cytochimiques spécifiques des polyphosphates. Experimental Cell Research, 15, 28-42.

- Ebel, J.P., Colas, J. & Muller, S. (1958b). Recherches cytochimiques sur les polyphosphates inorganiques contenus dans les organismes vivants. III. Presence de polyphosphates chez divers organismes inferieurs. Experimental Cell Research, 15, 36-42.
- Ebel, J.P. & Muller, S. (1958). Recherches cytochimiques sur les polyphosphates inorganiques contenus dans les organismes vivants. I. Etude de la reaction de metachromasie et des colorations au vert de methyle et à la pyronine en fonction de la longueur de chaine des polyphosphates. Experimental Cell Research, 15, 21-28.
- Eisenthal, R. & Cornish-Bowden, A. (1974). The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters. Biochemical Journal, 139, 715-720.
- Epstein, E. (1966). Dual pattern of ion absorption by plant cells and by plants. Nature, 212, 1324-1327.
- Epstein, E. & Elzam, O.E. (1963). Resolution of dual mechanisms of potassium absorption by barley roots. Proceedings of the National Academy of Science, U.S.A., 49, 684-692.
- Epstein, E. & Hagen, C.E. (1952). A kinetic study of the absorption of alkali cations by barley roots. Plant Physiology, 27, 457-474.
- Gianinazzi-Pearson, V. & Gianinazzi, S. (1976). Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza. I. Effect of mycorrhiza formation and phosphorus nutrition on soluble phosphatase activities in onion roots. Physiologie Vegetale, 14, 833-841.
- Gianinazzi-Pearson, V. & Gianinazzi, S. (1978) Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza. II. Soluble alkaline phosphatase specific to mycorrhizal infection in onion roots. Physiological Plant Pathology, 12, 45-53.
- Gianinazzi, S., Gianinazzi-Pearson, V. and Dexheimer, J. (1979). Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza. III. Ultrastructural localization of acid and alkaline phosphatase in onion roots infected by Glomus mosseae (Nicol. and Gerd.) The New Phytologist, 82, 127-132.
- Gimingham, C.H. (1972). Ecology of Heathlands. Chapman and Hall, London.
- Good, R. (1974). The Geography of the Flowering Plants, 4th edition, Longman, London.

- Goodman, J. & Rothstein, A. (1957). The active transport of phosphate into the yeast cell. Journal of General Physiology, 40, 915-921.
- Greenacre, M.J. (1984). Theory and Application of Correspondence Analysis. Academic Press, New York.
- Groves, R.H. (1983). Nutrient cycling in Australian Heath and South African Fynbos. In Ecological Studies Vol. 43. Mediterranean-Type Ecosystems : The Role of Nutrients (Ed. by F.J. Kruger, D.T. Mitchell & J.U.M. Jarvis), pp. 179-191. Springer-Verlag, Berlin.
- Harley, J.L. (1969). The Biology of Mycorrhiza 2nd Edition. Leonard Hill, London.
- Harley, J.L. & McCready, C.C. (1950) Uptake of phosphate by excised mycorrhizas of the beech. I. The New Phytologist, 49, 388-397.
- Harley, J.L. & McCready C.C. (1952). Uptake of phosphate by excised mycorrhizas of the beech. III. The effect of the fungal sheath on the availability of phosphate to the core. The New Phytologist, 51, 343-348.
- Harley, J.L. & McCready, C.C. (1981). Phosphate accumulation in Fagus mycorrhizas. The New Phytologist, 89, 75-80.
- Harley, J.L. & Smith, S.E. (1983). Mycorrhizal Symbiosis. Academic Press, London.
- Harold, F.M. (1962). Depletion and replenishment of the inorganic polyphosphate pool in Neurospora crassa. Journal of Bacteriology, 83, 1047-1057.
- Harold, F.M. (1966). Inorganic polyphosphates in biology : structure, metabolism and function. Bacteriological Reviews, 30(4), 772-794.
- Harold, F.M. & Sylvan, S. (1963). Accumulation of inorganic polyphosphate in Aerobacter aerogenes. II. Environmental control and the role of sulphur compounds. Journal of Bacteriology, 86, 222-231.
- Harrison, A.F. & Helliwell, D.R. (1979). A bioassay for comparing phosphorus availability in soils. Journal of Applied Ecology, 16, 497-505.
- Hasegawa, Y., Lynn, K.R. & Brockbank, W.J. (1976). Isolation and partial characterization of cytoplasmic and wall-bound acid phosphatases from wheat roots. Canadian Journal of Botany, 54, 1163-1169.

- Ho, I. and Zak, B. (1979). Acid phosphatase activity of six ectomycorrhizal fungi. Canadian Journal of Botany, 57,(11), 1203-1205.
- Hofstee, B.H.J. (1959). Non-inverted versus inverted plots in enzyme kinetics. Nature, 184, 1296-1298.
- Hughes, D.E. & Muhammed, A. (1962). The metabolism of polyphosphate in bacteria. Colloques International du Centre National de la Recherche Scientifique (Paris), 106, 591--602.
- Jennings, D.H. (1964). Changes in the size of orthophosphate pools in mycorrhizal roots of beech with reference to absorption of the ion from the external medium. The New Phytologist, 63, 181-193.
- Juni, E., Kamen, M.D., Spiegelman, S. & Wiame, J.M. (1947). Physiological heterogeneity of metaphosphate in yeast. Nature, 160, 717-718.
- Kempers, A.J. (1975). Determination of submicro-quantities of plant available phosphate in aqueous soil extracts. Plant and Soil, 42, 423-427.
- Kruger, F.J. (1979). South African Heathlands. In Heathlands of the World Vol. 9A. Heathlands and Related Shrublands : Descriptive Studies (Ed. by R.L. Specht), pp.19-80. Elsevier, Amsterdam.
- Lapeyrie, F.F., Chilvers, G.A. & Douglass, P.A. (1984). Formation of metachromatic granules following phosphate uptake by mycelial hyphae of an ectomycorrhizal fungus. The New Phytologist, 98, 345-360.
- Ling-Lee, M., Chilvers, G.A. & Ashford, A.E. (1975). Polyphosphate granules in three different kinds of tree mycorrhiza. The New Phytologist, 75, 551-554.
- Lowendorf, H.S., Slayman, C.L. & Slayman, C.W. (1974). Phosphate transport in Neurospora. Kinetic characterization of a constitutive, low-affinity system. Biochimica et Biophysica Acta, 373, 369-382.
- Lowendorf, H.S., Bazinet, G.F. & Slayman, C.W. (1975). Phosphate transport in Neurospora. Derepression of a high-affinity transport system during phosphorus starvation. Biochimica et Biophysica Acta, 389, 541-549.
- Lowry, O.H., Roseborough, N.J., Farr, A.L., & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193, 265-275.

- Martin, J.K. & Cartwright, B. (1971). The comparative plant availability of  $^{32}\text{P}$  myo-inositol hexaphosphate and  $\text{KH}_2^{32}\text{PO}_4$  added to soils. Communication of Soil Science and Plant Analysis, 2, 375-381.
- Martin, F., Canet, D., Rolin, D., Marchal, J.P. & Larher, F. (1983). Phosphorus -31 nuclear magnetic resonance study of polyphosphate metabolism in intact ectomycorrhizal fungi. Plant and Soil, 71, 469-476.
- McLennan, E.I. (1935). Non-symbiotic development of seedlings of Epacris impressa Labill. The New Phytologist, 34, 55-63.
- McNabb, R.F.R. (1961). Mycorrhiza in the New Zealand Ericales. Australian Journal of Botany, 9, 57-61.
- Mitchell, D.T. and Read, D.J. (1981). Utilization of inorganic and organic phosphates by the mycorrhizal endophytes of Vaccinium macrocarpon and Rhododendron ponticum. Transactions of the British Mycological Society, 76(2), 255-260.
- Mitchell, D.T., Brown, G. & Jongens-Roberts, S.M. (1984). Variation of forms of phosphorus in the sandy soils of coastal fynbos, south-western Cape. The Journal of Ecology, 72, 575-584.
- Mitchell, D.T. & Read, D.J. (1985). Growth of African and European ericoid mycorrhizal endophytes on a range of substrates. Transactions of the British Mycological Society, 84, 355-357.
- Mitchell, D.T., Coley, P.G.F., Webb, S. & Allsopp, N. (in press). Litter fall and decomposition processes in the coastal fynbos vegetation, south-western Cape, South Africa.
- Moll, E.J. & Jarman, M.L. (1984), Clarification of the term fynbos. South African Journal of Science, 80(8), 351-352.
- Muller, S. & Ebel, J.P. (1958). Études des polyphosphates condensés contenus dans divers champignons. Bulletin de la société de chimie biologique, 40, 1153-1161.
- Murphy, J. & Riley, J.P. (1962). A modified single solution method for the determination of phosphate in natural waters. Analytica Chimica Acta, 27, 31-36.
- Neame, K.D. & Richards, T.G. (1972). Elementary Kinetics of Membrane Carrier Transport, pp.80-90. Blackwell Scientific Publications, Oxford.



- Nissen, Per. (1973). Kinetics of ion uptake in higher plants. Physiologia Plantarum, 28, 113-120.
- Nissen, Per. (1974). Uptake mechanisms : inorganic and organic. Annual Review of Plant Physiology, 25, 53-79.
- Nye, P.H. & Tinker, P.B. (1977). Solute Movement in the Soil-Root System. Blackwell Scientific Publications, Oxford.
- Pearson, V. & Read, D.J. (1973a). The biology of mycorrhiza in the Ericaceae. I. The isolation of the endophyte and synthesis of mycorrhizas in aseptic culture. The New Phytologist, 72, 371-379.
- Pearson, V.J. & Read, D.J. (1973b). The biology of mycorrhiza in the Ericaceae. II. The transport of carbon and phosphorus by the endophyte and the mycorrhiza. The New Phytologist, 72, 1325-1331.
- Pearson, V. & Read, D.J. (1975). The physiology of the mycorrhizal endophyte of Calluna vulgaris. Transactions of the British Mycological Society, 64, 1-7.
- Peterson, T.A., Mueller, W.C. & Englander, L. (1980). Anatomy and ultrastructure of a Rhododendron root-fungus association. Canadian Journal of Botany, 58(23), 2421-2435.
- Pierce, S.M. (1984). A synthesis of plant phenology in the Fynbos Biome. South African National Scientific Programmes Report No. 88.
- Read, D.J. (1978). The biology of mycorrhiza in heathland ecosystems with special reference to nitrogen nutrition of the Ericaceae. In Microbial Ecology : International Microecology Symposium, New Zealand (Ed. by M.W. Loutit & J.A.R. Miles), pp. 324-328. Springer-Verlag, Berlin.
- Read, D.J. (1983). The biology of mycorrhiza in the Ericales. Canadian Journal of Botany, 61, 985-1004.
- Read, D.J. & Mitchell, D.T. (1983). Decomposition and mineralization processes in mediterranean-type ecosystems and in heathlands of similar structure. In Ecological Studies Vol. 43. Mediterranean-Type Ecosystems : The Role of Nutrients (Ed. by F.J. Kruger, D.T. Mitchell & J.U.M. Jarvis), pp. 208-232. Springer-Verlag, Berlin.
- Reid, M.S. & Bielecki, R.L. (1968). A simple apparatus for vertical flat-sheet polyacrylamide gel electrophoresis. Analytical Biochemistry, 22, 374-381.

- Robbins, W.J. & White, V.B. (1936). Limited growth and abnormalities in excised corn root tips. Botanical Gazette, 98, 209-242.
- Rolin, D., Le Taçon, F. & Larher, F. (1984). Characterization of the different forms of phosphorus in the mycelium of the ectomycorrhizal fungus, Hebeloma cylindrosporum, cultivated in pure culture. The New Phytologist, 98, 335-343.
- San Blas, G. & Cunningham, W.L. (1974). Production of extracellular acid phosphatase by the yeast Hansenula holstii NCYC 560. Biochimica et Biophysica Acta, 343, 208-210.
- Schmidt, G. (1961). Nonspecific acid phosphomonoesterases. In The Enzymes Vol. 5., 2nd edition. (Ed. by Boyer, P.D., Lardy, H. & Myrback, K.), pp. 37-47. Academic Press, New York.
- Schmidt, G. & Thannhauser, S.J. (1945). A method for the determination of deoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. The Journal of Biological Chemistry, 162, 83-89.
- Specht, R.L. (1979). Heathlands and related shrublands of the world. In Heathlands of the World, Vol. 9A. Heathlands and Related Shrublands : Descriptive Studies (Ed. by R.L. Specht), pp. 1-18. Elsevier, Amsterdam.
- Specht, R.L., Moll, E.J., Pressinger, F. and Sommerville, J. (1983). Moisture regime and nutrient control of seasonal growth in mediterranean ecosystems. In : Ecological Studies Vol. 43. Mediterranean-Type Ecosystems : The Role of Nutrients (Ed. by F.J. Kruger, D.T. Mitchell & J.U.M. Jarvis), pp. 120-132. Springer-Verlag, Berlin.
- Spiers, G.A. & McGill, W.B. (1979). Effects of phosphorus addition and energy supply on acid phosphatase production and activity in soils. Soil Biology and Biochemistry, 11, 3-8.
- Stock, W.D. & Lewis, O.A.M. (1986). Soil nitrogen and the role of fire as a mineralizing agent in a South African coastal fynbos ecosystem. Journal of Ecology (in press).
- Stock, W.D., Lewis, O.A.M. & Allsopp, N. (1986). Soil nitrogen mineralization in a post-fire coastal fynbos succession at Pella, south-western Cape, South Africa. Oecologia (in press).

- Stribley, D.P. & Read, D.J. (1974a). The biology of mycorrhiza in the Ericaceae. III. Movement of carbon-14 from host to fungus. The New Phytologist, 73, 731-741.
- Stribley, D.P. & Read, D.J. (1974b). The biology of mycorrhiza in the Ericaceae. IV. The effects of mycorrhizal infection on uptake of  $^{15}\text{N}$  from labelled soil by Vaccinium macrocarpon Ait. The New Phytologist, 73, 1149-1155.
- Stribley, D.P. & Read, D.J. (1976). The biology of mycorrhiza in the Ericaceae. VI. The effects of mycorrhizal infection and concentration of ammonium nitrogen on growth of cranberry (Vaccinium macrocarpon Ait.) in sand culture. The New Phytologist, 77, 63-72.
- Stribley, D.P. and Read, D.J. (1980). The biology of mycorrhiza in the Ericaceae. VII. The relationship between mycorrhizal infection and the capacity to utilise simple and complex organic nitrogen sources. The New Phytologist, 86, 365-371.
- Strullu, D.G., Gourret, J.P., Garrec, J.P. & Fourcy, A. (1981). Ultrastructure and electron-probe microanalysis of the metachromatic vacuolar granules occurring in Taxus mycorrhizas. The New Phytologist, 87, 537-545.
- Strullu, D.G., Harley, J.L., Gourret, J.P. and Garrec, J.P. (1982). Ultrastructure and microanalysis of the polyphosphate granules of the ectomycorrhizas of Fagus sylvatica. The New Phytologist, 92, 417-423.
- Strullu, D.G., Harley, J.L., Gourret, J.P. & Garrec, J.P. (1983). A note on the relative phosphorus and calcium contents of metachromatic granules in Fagus mycorrhizas. The New Phytologist, 94, 89-94.
- Takhtajan, A. (1969). Flowering Plants : Origin and Dispersal. Oliver and Boyd, Edinburgh.
- Theodorou, C. (1968). Inositol phosphate in needles of Pinus radiata D. Don and the phytase activity of mycorrhizal fungi. Proceedings of the Congress of Soil Science Adelaide, 3, 483-493.
- Theodorou, C. (1971). The phytase activity of the mycorrhizal fungus Rhizopogon roseolus. Soil Biology and Biochemistry, 3, 89-90.

- Thomas, G.W., Clarke, C.A., Mosse, B. and Jackson, R.M. (1982). Source of phosphate taken up from two soils by mycorrhizal (Thelephora terrestris) and non-mycorrhizal Picea sitchensis seedlings. Soil Biology and Biochemistry, 14, 73-75.
- Tinker, P.B. (1975). The soil chemistry of phosphorus and mycorrhizal effects on plant growth. In Endomycorrhizas (Ed. by F.E. Sanders, B. Mosse & P.B. Tinker), pp. 353-371. Academic Press, London.
- Verjee, Z.H.M. (1969). Isolation of three acid phosphatases from wheat germ. European Journal of Biochemistry, 9, 439-444.
- White, J.A. and Brown, M.F. (1979). Ultrastructure and X-ray analysis of phosphorus granules in a vesicular-arbuscular mycorrhizal fungus. Canadian Journal of Botany, 57(24), 2812-2818.
- Wiame, J.M. (1949). The occurrence and physiological behaviour of two metaphosphate fractions in yeast. Journal of Biological Chemistry, 178, 919-929.
- Williamson, B. & Alexander, I.J. (1975). Acid phosphatase localised in the sheath of beech mycorrhiza. Soil Biology and Biochemistry, 7, 195-198.
- Woolhouse, H.W. (1969). Differences in the properties of the acid phosphatases of plant roots and their significance in the evolution of adaptive ecotypes. In Ecological Aspects of Mineral Nutrition of Plants (Ed. by I.H. Robinson). Symposium of the British Ecological Society, 9, 357-380.
- Woolhouse, H.W. (1975). Membrane structure and transport problems considered in relation to phosphorus and carbohydrate movements and the regulation of endotrophic mycorrhizal associations. In Endomycorrhizas (Ed. by R.E. Sanders, B. Mosse, and P.B. Tinker), pp. 209-239. Academic Press, London.
- Zink, M.W. and Veliky, I.A. (1979). Acid phosphatases of Ipomoea sp. cultured in vitro. I. Influence of pH and inorganic phosphate on the formation of phosphatases. Canadian Journal of Botany, 57, 739-753.

**RAPS-**



PAGE INDEX

**RAPS-**



PAGE INDEX

## APPENDIX I

```

10 DIM X1(18),Y1(18)
20 DIM X(10),Y(10),A(50),B(50)
30 DEF FNA(Z)=V*Z/(K+Z)
40 CLS
50 PRINT SPC(10);"DOUBLE HYPERBOLA CURVE FIT"
60 PRINT : PRINT
70 *****
80 PRINT "HOW MANY DATA PAIRS : " : INPUT M
90 PRINT "ENTER THE";M;" DATA PAIRS, CONC FIRST, FROM LOWEST CONC"
100 FOR I=1 TO M : INPUT X1(I) : PRINT SPC(5); : INPUT Y1(I)
110 NEXT I
120 PRINT "PARTITION OCCURS AFTER DATA PAIR : " : INPUT P
125 PRINT "WORKING"
130 L1=1 : L2=1 : L3=1 : L4=1
140 FOR Q=1 TO 10
150 FOR I=1 TO P : X(I)=X1(I)
160 Y(I)=Y1(I)-FNA(X1(I)) : NEXT I
170 Z=P : GOSUB 490
180 FOR I=P+1 TO M : X(I-P)=X1(I)
190 Y(I-P)=Y1(I)-FNA(X1(I)) : NEXT I
200 Z=M-P : GOSUB 490
210 C1=ABS((K-L1)/L1) : C2=ABS((V-L2)/L2)
220 C3=ABS((K2-L3)/L3) : C4=ABS((V2-L4)/L4)
230 IF C2>C1 THEN 240
240 C1=C2
250 IF C3>C1 THEN 260
260 C1=C3
270 IF C4>C1 THEN 280
280 C1=C4
290 IF C1<.0001 THEN 320
300 L1=K : L2=V : L3=K2 : L4=V2
310 NEXT Q
320 C2=0
330 CLS
340 LPRINT : LPRINT SPC(10);"PARTITION METHOD 'I' FIT" : LPRINT
350 LPRINT "PARTITION MADE AFTER DATA PAIR: ";P
360 LPRINT
370 LPRINT "X(CONC)","Y UPTAKE","Y PRED";SPC(10);"ERROR"
380 FOR I=1 TO M : Z=X1(I)
390 C3=FNA(Z)+V2*Z/(K2+Z)
400 'PRINT Z,Y1(I),C3;SPC(9);"%-";
405 LPRINT USING "##.####";Z;
406 LPRINT SPC(7);: LPRINT USING "##.####";Y1(I);
407 LPRINT SPC(7);: LPRINT USING "##.####";C3;: LPRINT SPC(6);"%-";
410 LPRINT USING "####.####";Y1(I)-C3
420 ***
430 C2=C2+(Y1(I)-C3)^2
440 NEXT I
445 LPRINT
450 LPRINT "KM1= " : LPRINT USING "##.####";K;: LPRINT " VMAX1= " : LPRINT USING
"##.####";V
460 LPRINT "KM2= " : LPRINT USING "##.####";K2;: LPRINT " VMAX2= " : LPRINT USIN
G "##.####";V2

```

```

465 LPRINT
470 LPRINT "ITERATIONS=";Q;"RESIDUAL SUM OF SQUARES=";C2
475 LPRINT CHR$(12);
480 GOTO 710
490 K2=K : V2=V : K=0
495 PRINT "***";
500 FOR I=1 TO Z-1 : R=X(I) : S=Y(I)
510 FOR J=I+1 TO Z : IF X(J)=R THEN 540
520 D=S*X(J)-R*Y(J) : IF D=0 THEN 540
530 K=K+1 : A(K)=(Y(J)-S)*X(J)*R/D : B(K)=S*(A(K)/R+1)
540 NEXT J : NEXT I
550 L=K-1
560 J=1 : FOR I=1 TO L
570 IF A(I+1)>=A(I) THEN 590
580 D=A(I) : A(I)=A(I+1) : A(I+1)=D : J=I
590 NEXT I
600 L=J-1 : IF L>0 THEN 560
610 L=K-1
620 J=1 : FOR I=1 TO L
630 IF B(I+1)>=B(I) THEN 650
640 D=B(I) : B(I)=B(I+1) : B(I+1)=D : J=I
650 NEXT I
660 L=J-1 : IF L>0 THEN 620
670 IF INT(K/2)*2=K THEN 690
680 I=INT(K/2)+1 : R=A(I) : S=B(I) : GOTO 700
690 I=INT(K/2) : R=(A(I)+A(I+1))/2 : S=(B(I)+B(I+1))/2
700 K=R : V=S : RETURN
710 CLS
720 PRINT SPC(10);"DOUBLE HYPERBOLA CURVE FIT"
730 PRINT : PRINT
740 INPUT "DO YOU WISH TO TRY ANOTHER PARTITION "; ANS$
750 IF ANS$="Y" THEN PRINT : GOTO 120
760 IF ANS$<>"N" THEN 710
770 END

```

*New Phytol.* (1985) **99**, 431–440

431

## THE CHARACTERIZATION AND ESTIMATION OF POLYPHOSPHATES IN ENDOMYCORRHIZAS OF THE ERICACEAE

BY C. J. STRAKER AND D. T. MITCHELL

*Department of Botany, University of Cape Town, Rondebosch 7700, South Africa*

(Accepted 28 September 1984)

### SUMMARY

Metachromatic staining demonstrated the presence of polyphosphate granules in endophytes isolated from root systems of *Vaccinium macrocarpon* Ait., *Rhododendron ponticum* L., *Calluna vulgaris* (L.) Hull, *Erica hispidula* L. and *E. mauritanica* L. The granules accumulated in response to high concentrations of phosphorus in the external medium and during the lag phase of growth. Nucleic acid-polyphosphate co-precipitates prepared from endophytes were separated by means of polyacrylamide gel electrophoresis and the molecular weights of polyphosphate of three endophytes were between 3000 and 4700. Inoculated root systems of *Vaccinium macrocarpon* had significantly more acid-labile polyphosphate than non-mycorrhizal roots. The results are compared with studies of polyphosphate in ecto- and vesicular-arbuscular mycorrhizas and are discussed in relation to ultrastructural analyses and the role of polyphosphate in the phosphorus nutrition of ericaceous plants in heathlands.

Key words: Polyphosphate, ericoid mycorrhizas

### INTRODUCTION

Studies on polyphosphate (polyP) granules in mycorrhizal systems have concentrated on vesicular-arbuscular (Cox *et al.*, 1975; White & Brown, 1979) and ecto-mycorrhizas (Ashford, Ling-Lee & Chilvers, 1975; Ling-Lee, Chilvers & Ashford, 1975; Strullu *et al.*, 1981; Strullu *et al.*, 1982, 1983). The granules from these systems have been implicated in both storage (Chilvers & Harley, 1980; Harley & McCready, 1981) and translocation of phosphorus (Callow *et al.*, 1978; Cox *et al.*, 1980). If the electron-dense, osmiophilic granules identified in ultrastructural analyses of ericoid mycorrhizas by Bonfante-Fasola & Gianinazzi-Pearson (1979), Peterson, Mueller & Englander (1980), Bonfante-Fasola & Gianinazzi-Pearson (1981), Bonfante-Fasola, Berta & Gianinazzi-Pearson (1982) and Duddridge & Read (1982) are rich in phosphorus, they may be of fundamental importance in the phosphorus nutrition of ericaceous plants especially when situated under conditions of low phosphorus status.

This paper confirms the presence of polyP in pure cultures of ericoid mycorrhizal fungi and indicates the potential of the granules to be a phosphorus sink under conditions of abundant external phosphorus supply. The identification and characterization of polyP are based upon histochemical and biochemical techniques and the results are compared with other studies undertaken on vesicular-arbuscular and ectomycorrhizas.



## MATERIALS AND METHODS

*Isolation of endophytes*

Mycorrhizal endophytes of *Vaccinium macrocarpon* Ait., *Rhododendron ponticum* L. and *Calluna vulgaris* (L.) Hull from the United Kingdom and *Erica hispidula* L. and *E. mauritanica* L. from South Africa were isolated from root systems using the serial washing and maceration techniques described by Pearson & Read (1973). Cultures of *V. macrocarpon* and *R. ponticum* were those used by Mitchell & Read (1981) whereas the endophyte of *C. vulgaris* was isolated from seedlings taken from Parys Mountain, Anglesey, United Kingdom. Seedlings of *E. hispidula* and *E. mauritanica* were growing in acid Table Mountain sandstone soils at the National Botanic Gardens, Kirstenbosch and Tokai forest respectively, 15 to 18 km S.E. of Cape Town. All the endophytes were grown on 2% malt extract agar. The South African isolates have been successfully back-inoculated into seedlings of *V. macrocarpon* and re-isolated.

*Preparation of cultures*

The basal liquid nutrient medium was similar to that used by Mitchell & Read (1981) with the addition of 50 mg dm<sup>-3</sup> yeast extract. When the P source was organic (sodium inositol hexaphosphate), 2 cm<sup>3</sup> of the P solution was passed through a "Millipore" filter (0.45 µm) and added to 18 cm<sup>3</sup> of the basal medium (autoclaved at 115 °C for 20 min) in 100 cm<sup>3</sup> Pyrex bottles. Inorganic-P (Na<sub>2</sub>HPO<sub>4</sub>) was added directly to the basal medium before autoclaving. The pH of the culture media was adjusted to 7.0 with the addition of sterile 0.1 M HCl.

Inocula were prepared by removing marginal segments of mycelium from agar cultures and homogenizing these in 10 cm<sup>3</sup> of sterile distilled water. One loop of homogenate was then transferred to each of the culture bottles. The cultures were incubated at 25 °C under static conditions.

*Cytochemical methods for the identification of polyphosphates*

PolyP granules were observed in the hyphae of endophytes by using the staining and extraction techniques of Ashford *et al.* (1975) and Ling-Lee *et al.* (1975).

*Phenol-detergent extraction of undegraded nucleic acid-polyP co-precipitates*

Extracts of mycelial cultures and seedlings, obtained by the method described by Callow *et al.* (1978) were dissolved in 0.5 cm<sup>3</sup> 10% sucrose in 0.01 M Tris-HCl buffer (pH 7.8). Ribonucleic acid (RNA) in samples of the extracts were adsorbed on to activated charcoal by the method of Bennet & Scott (1971) before incubation for 10 min at 100 °C in 1 M HCl to hydrolyze the acid-labile polyP. Total P was then assayed by the colorimetric method of Kempers (1975).

*Polyacrylamide gel electrophoresis*

Polyphosphates and nucleic acids were separated by gel electrophoresis. The method was similar to that used by Callow *et al.* (1978) except that electrophoresis was performed on 8.5% (w/v) acrylamide gels using a vertical flat-bed apparatus similar to that described by Reid & Bielecki (1968). After pre-electrophoresing for 2 h at 10 mA constant current, 30 µl samples were loaded on to the gel and run for 15 min at 15 mA followed by up to 2 h at 30 mA. Gels were run at 10 °C, then stained by immersion in 0.1% toluidine blue in 1% acetic acid. After staining and

destaining, individual pink (polyP) and blue (nucleic acid) bands were scanned between 500 and 700 nm in a Pye Unicam SP1800 spectrophotometer. Gels were scanned using a Vitatron densitometer at fixed wavelengths closest to absorption maxima.

#### *Molecular weight determinations of polyP*

An extract (30  $\mu$ l) was run on an 8.5% gel with a range of synthetic sodium polyP compounds (sodium phosphate glasses,  $\text{Na}_{n+2}\text{P}_n\text{O}_{3n+1}$  Types 35, 45, 65, 135; Sigma Chemical Co.) of known molecular weights. The logarithmic relationship between the distance of migration (determined from densitometer scans) and molecular weight of the markers was expressed in the form of a power curve equation.

#### *Total P determinations*

Mycelial cultures, seedlings and pink-staining gel segments were digested with a tri-acid mixture (10 parts  $\text{HNO}_3$ : 1 part  $\text{H}_2\text{SO}_4$ : 4 parts  $\text{HClO}_4$ ) at 150 to 180 °C. Total P was assayed by the method of Kempers (1975).

#### *Synthesis of mycorrhizal root systems*

Seeds extracted from fresh fruits of *P. macrocarpon* were surface sterilized in 3% sodium hypochlorite for 5 min, washed thoroughly with sterile, distilled water and transferred to plates of 1% agar. After three weeks seedlings were transferred to McCartney bottles containing 20 cm<sup>3</sup> of the following autoclaved medium (Robbins & White, 1936):  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 10 mg;  $\text{KH}_2\text{PO}_4$ , 10 mg;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 2 mg;  $\text{NH}_4\text{Cl}$ , 32 mg;  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 33.5 mg; agar, 10.0 g with distilled water to 1 dm<sup>3</sup>, supplemented with 0.5 g dm<sup>-3</sup> glucose, 1 g dm<sup>-3</sup> activated charcoal (Duclos & Fortin, 1983) and covered with a thin layer of sterile acid-washed sand when set. The uncapped bottles were placed in sterile containers consisting of glass boxes (47 cm  $\times$  32.6 cm  $\times$  20 cm high) standing in stainless steel trays, which were placed in growth cabinets with 16 h daylight at 20 °C and 8 h darkness at 15 °C and an irradiance of 30 Wm<sup>-2</sup>.

After six weeks, the lightly infected seedlings were transferred to moist, sterile Clovelly soil and grown for another six weeks under the same conditions by which time infection of the root systems was sufficiently developed to permit harvesting of the seedlings. All root systems were thoroughly washed under tap water and in a number of changes of distilled water prior to either extraction and digestion procedures or re-isolation of the endophytes.

#### *Statistical analysis*

Size classes of metachromatic granules were obtained from three separate cultures, each culture representing 40 to 80 random measurements from younger, marginal hyphae. Percentages were converted to arcsin transformations. The original measurements were subjected to a computer-based programme of Correspondence Analysis (Greenacre, 1984). All other granule values were obtained from 3 separate cultures, each culture representing 10 to 20 random counts. Values given for the phenol-detergent extraction of the *E. hispidula* endophyte represent three separate extractions; all other values represent at least three replicates from a single extraction. Molecular weights of polyP molecules were determined from four separate gels for each of the three endophytes used.

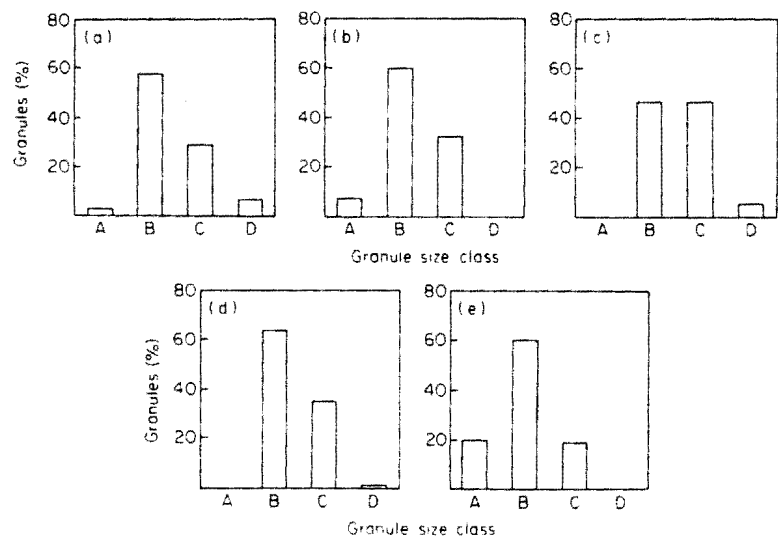


Fig. 1. The percentage of metachromatic granules in relation to different size classes in 10-d-old mycelia of endophytes isolated from root systems of (a) *Calluna vulgaris*, (b) *Vaccinium macrocarpon*, (c) *Rhododendron ponticum*, (d) *Erica hispidula*, (e) *Erica mauritanica* and grown on basal medium and 3.23 mM sodium phytate. (Size classes are based on diameter of granules as follows: A, < 0.5  $\mu$ m; B, 0.5  $\mu$ m to 1.0  $\mu$ m; C, 1.0  $\mu$ m to 1.5  $\mu$ m; D, > 1.5  $\mu$ m.)

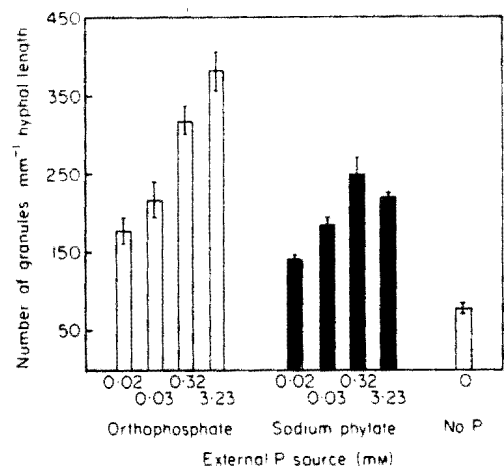


Fig. 2. Numbers of metachromatic granules in endophyte of *Vaccinium macrocarpon* grown for 14 d in liquid culture with various sources of P. Vertical bars represent twice SE.

RESULTS

Cytochemical observation of polyP granules

A positive metachromatic reaction was obtained when hyphae were stained with toluidine blue at pH 1.0 and granules contained within vacuoles were readily observed in young hyphae. In older hyphae, vacuoles were larger and the granules were not easily identifiable. Figure 1 shows the range of size classes for five different endophytes. Correspondence Analysis revealed that granules of *E. mauritanica* were smaller than those of the other endophytes under similar growing

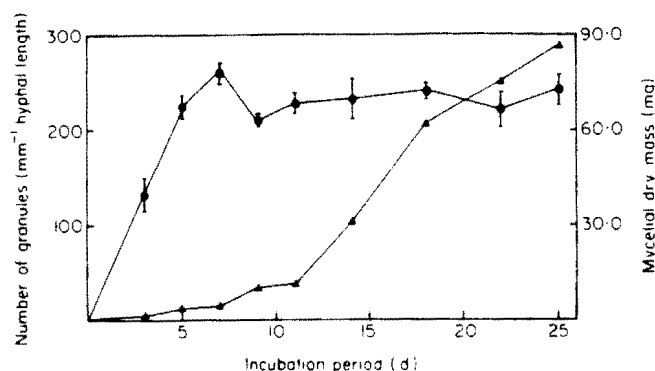


Fig. 3. Frequency of metachromatic granules during growth of the endophyte of *Vaccinium macrocarpon* (● — ●, granule numbers; ▲ — ▲, mycelial dry mass). Basal medium initially supplied with 3.23 mM sodium phytate. Vertical bars represent twice SE.

conditions. When material was stained with lead nitrate followed by ammonium sulphide, granules similar in size, frequency and distribution to metachromatic granules were stained black.

Numbers of metachromatic granules could be used as a measure of concentration of polyP and Figure 2 shows their relationship to the external concentration of phosphorus in the medium. When the P source was orthophosphate, there was a direct power curve relationship between these variables ( $y = 155.6x^{0.11}$  with  $r^2 = 0.94$ ). However, when P was supplied in an organic form, granule numbers increased up to an external concentration of 0.32 mM but thereafter they declined. Granules in hyphae during the growth of the endophyte in culture accumulated rapidly during the lag phase of growth (Fig. 3).

#### *Polyacrylamide gel electrophoresis for separation of nucleic acid-polyP co-precipitates*

The pink-staining bands obtained from mycelial extracts of a South African and European isolate as well as commercial polyP (sodium phosphate glass Type 65) have absorption maxima at 530 nm ( $\gamma$ -metachromasy) (Fig. 4). Blue metachromatic gel bands from mycelial extracts and commercial RNA showed a shift to an absorption maximum of 580 nm ( $\beta$ -metachromasy) (Fig. 4). The  $\gamma$ -metachromasy of the pink bands produced absorption peaks at 523 nm whereas the major blue bands were enhanced at 577 nm (Fig. 5).

#### *Molecular weights of polyP molecules*

Electrophoretic bands of a range of synthetic polyP markers were run on the same gel plate as the mycelial extracts (Fig. 5). The relationship between distance of migration and molecular weight is expressed by the following power curve equations:

$$y = 27006.2x^{-0.6546} \text{ with } r^2 \text{ of } 0.77 \text{ for the } E. hispidula \text{ endophyte.}$$

$$y = 1357375.4x^{-1.693} \text{ with } r^2 \text{ of } 0.83 \text{ for the } E. mauritanica \text{ endophyte.}$$

$$y = 389942.0x^{-1.435} \text{ with } r^2 \text{ of } 0.98 \text{ for the } R. ponticum \text{ endophyte.}$$

The molecular weights of the polyP of the above endophytes were estimated to be  $4663 \pm 136$ ,  $4131 \pm 231$  and  $2978 \pm 288$ , respectively.

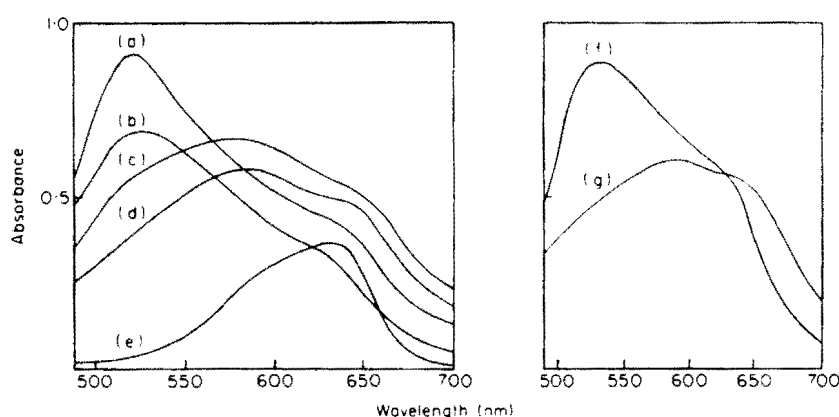


Fig. 4. Absorption spectra of segments of 8.5% polyacrylamide gels stained with 0.1% toluidine blue: (a) Pink-staining metachromatic band from extracts of endophyte of *Erica hispidula*; (b) Pink-staining metachromatic band from gel on which 50  $\mu$ g synthetic polyP had been run; (c) A blue-staining nucleic acid band from extracts of endophyte of *E. hispidula*; (d) A blue-staining band from gel on which 50  $\mu$ g commercial RNA had been run; (e) A segment of gel stained only with toluidine blue; (f) Pink-staining metachromatic band from extracts of endophyte of *Rhododendron ponticum*; (g) A blue-staining nucleic-acid band from extracts of endophyte of *R. ponticum*.

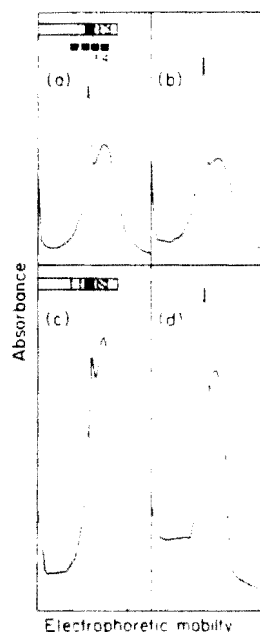


Fig. 5. Scans of nucleic acid-polyP phenol-detergent extracts separated on 8.5% polyacrylamide gel and stained with 0.1% toluidine blue: (a) Gel of extract of endophyte of *Rhododendron ponticum* scanned at 523 nm; (b) Same gel as in (a) scanned at 577 nm; (c) Gel of extract of endophyte of *Erica hispidula* scanned at 523 nm; (d) Same gel as in (c) scanned at 577 nm. Diagrammatic representations of the bands are shown above the scans. Solid areas are the blue, nucleic acid components; the cross-hatched area represents the pink, polyP component. Also shown are positions of synthetic polyP markers in relation to scans: (1) Type 135 (chain length 132); (2) Type 65 (chain length 65); (3) Type 45 (chain length 46); (4) Type 35 (chain length 39).

P and acid-soluble polyP content

The total P and acid-soluble polyP content of the endophytes of *E. hispidula* and *R. ponticum* were similar (the latter being 11 and 8%, respectively of total P) (Table 1). The endophyte of *E. mauritanica* showed a higher acid-soluble polyP content but this formed a smaller proportion (7%) of a much higher total P content. The similarity between the acid-soluble polyP content of the extracts and the total P of the pink, metachromatic gel bands confirmed the presence of a

Table 1. Acid-labile polyP content of phenol-detergent extracts and total P of isolated endophytes grown in culture on basal medium and 3.23 mM sodium phytate for 8 d and aseptically infected root systems of *Vaccinium macrocarpon*

	Fresh mass $\pm$ SE ( $\mu$ mol g <sup>-1</sup> )			(%) of total
	Acid-labile P	Total P of pink bands	Total P	Acid-labile P
Endophyte				
<i>E. hispidula</i>	1.9 $\pm$ 0.1	1.7 $\pm$ 0.3	17.5 $\pm$ 2.0	10.9
<i>R. ponticum</i>	1.4 $\pm$ 0.3	1.1 $\pm$ 0.2	18.2 $\pm$ 2.1	7.7
<i>E. mauritanica</i>	2.2 $\pm$ 0.2	2.1 $\pm$ 0.5	33.6 $\pm$ 1.4	6.6
Root systems				
Infected with				
<i>E. hispidula</i> endophyte	1.0 $\pm$ 0.2 <sup>a</sup>	—	9.5 $\pm$ 2.0	10.5
Infected with				
<i>E. mauritanica</i> endophyte	3.9 $\pm$ 0.3 <sup>b</sup>	—	19.8 $\pm$ 3.2 <sup>a</sup>	19.7
Uninfected	0.3 $\pm$ 0.1	—	10.4 $\pm$ 2.6	3.0

a and b are significantly different from uninfected root at 0.05 and 0.001 level respectively; — signifies not determined.

predominately acid-soluble fraction. Root systems of seedlings of *V. macrocarpon* were inoculated with the endophytes of *E. hispidula* and *E. mauritanica* and, after three months incubation, the proportions of acid-labile polyP of total P were 10 and 20%, respectively. Infection with the *E. mauritanica* endophyte also resulted in a two-fold increase in the total P of the root system when compared with uninfected roots, which had a low acid-labile P content of 3% of the total P. Infection with the endophyte of *E. hispidula* did not result in a similar increase in the total P of the root system.

DISCUSSION

Phosphate appears to be a limiting element for growth in heathlands, the natural habitat of ericaceous plants. These include the acidic sandy soils with a low organic matter content from S.W. Cape, South Africa and the acidic mor-humus soils with a high organic matter content from Europe (Read & Mitchell, 1983). An investigation of the phosphate metabolism of these plants is therefore an essential step towards an understanding of their ecology.

The presence of polyP in pure cultures of the endophyte and mycorrhizal root systems of ericaceous seedlings has been confirmed. The metachromatic granules resemble closely those of ectomycorrhizas of eucalypts and pines (Ashford *et al.*,

1975; Ling-Lee *et al.*, 1975; Chilvers & Harley, 1980) and vesicular-arbuscular mycorrhizas (Cox *et al.*, 1975). The range of granule sizes were similar to those obtained for beech mycorrhizas incubated in 1 mM orthophosphate (Chilvers & Harley, 1980) and granule numbers were sensitive to both the form and concentration of the external P source (Fig. 2). The decline in granule numbers after an organic P concentration of 0.32 mM may be due to the end-product inhibition of the phosphatase enzymes (Pearson & Read, 1975) which would have reduced the amount of P available for uptake. The accumulation of granules in hyphae in media without the addition of an external P source was most likely due to the presence of residual P in the yeast extract of the basal medium. The polyP granules accumulated during the lag phase of growth. In *Corynebacterium xerosis*, numbers of granules increased during the lag phase but declined during the exponential phase (Hughes & Muhammed, 1962).

The phenol-detergent extraction technique of Callow *et al.* (1978) was used to isolate polyP and nucleic acid in a relatively undegraded form and the polyP was finally characterized by means of polyacrylamide gel electrophoresis. Callow *et al.* (1978) were able to determine the molecular weight and chain length of the polyP in vesicular-arbuscular mycorrhizas as polyphosphates have a constant charge to mass ratio and their mobility in the gels is a function of their molecular size. In this study, the molecular weights of 3000 to 4700 from ericoid endophytes were considerably lower than the figure of 20800 for polyphosphates of vesicular-arbuscular mycorrhizal fungi (Callow *et al.*, 1978) although the possibility of chain degradation during extraction should not be discounted. The proportion of acid-labile polyP to total P was 7 to 11% in the cultured endophyte. Isolated internal vesicular-arbuscular endophytes have been estimated to contain 16% total polyP (Capaccio & Callow, 1982). Ecto-mycorrhizas incorporated 40% of absorbed  $^{32}\text{P}$  into total polyP over 2.5 h (Harley & McCready, 1981) although clearly this does not represent a steady state condition. The polyP content of ericoid mycorrhizal root systems was significantly greater than that of the non-mycorrhizal controls indicating the polyP to be largely fungal in origin. The higher proportion of polyP in the root systems inoculated with the endophyte from *E. mauritanica* could have been due to a heavier mycorrhizal infection observed. However, as the higher total P content of these infected seedlings was correlated with a high concentration of P in the isolated endophyte, the mycorrhizal fungus of *E. mauritanica* may also be able to accumulate more phosphorus. Thus, preliminary results suggest that the capacity of the endophyte to store polyP may play an important part in the phosphorus nutrition of ericaceous plants. This is emphasized by the increase in granules of the endophyte in culture concomitant with the increase in concentration of the external supply of orthophosphate.

Electron-dense granules are located in the fungal vacuoles in both the cultured endophyte and extra- and intracellular hyphae of natural ericoid mycorrhizal root systems (Bonfante-Fasola & Gianinazzi-Pearson, 1981). However, in dual cultures, they often occur in the external mycelium but rarely in intracellular hyphae (Bonfante-Fasola & Gianinazzi-Pearson, 1982). It appears that the storage of phosphorus as polyP in the endophyte would not persist and transfer from the fungus to the host may involve an active process. The mycorrhizal endophyte outlives the ericaceous cortical cell in which cytoplasmic contents increase with infection indicating a mycorrhizal association of intense metabolic activity (Bonfante-Fasola & Gianinazzi-Pearson, 1982; Duddridge & Read, 1982; Read, 1983). Biochemical and histochemical evidence and ultrastructural studies indicate

that the endophyte exterior to the root exploits the soil for phosphate, part of which accumulates in these external hyphae as vacuolar granules of polyP molecules.

#### ACKNOWLEDGEMENTS

We wish to thank the CSIR, South Africa and the University of Cape Town for financial assistance, Dr T. T. Dunne for assistance with the Correspondence Analysis and Dr D. J. Read of Sheffield University for the use of the ericoid endophytes from the UK.

#### REFERENCES

- ASHFORD, A. E., LING-LEE, M. & CHILVERS, G. A. (1975). Polyphosphate in eucalypt mycorrhizas: a cytochemical demonstration. *The New Phytologist*, **74**, 447-453.
- BONFANTE-FASOLA, P. & GIANINAZZI-PEARSON, V. (1979). Ultrastructural aspects of endomycorrhiza in the Ericaceae. I. Naturally infected hair roots of *Calluna vulgaris* L. Hull. *The New Phytologist*, **83**, 739-744.
- BONFANTE-FASOLA, P. & GIANINAZZI-PEARSON, V. (1981). Ultrastructural aspects of endomycorrhizas in the Ericaceae. II. Host-endophyte relationships in *Vaccinium myrtillus* L. *The New Phytologist*, **89**, 219-224.
- BONFANTE-FASOLA, P. & GIANINAZZI-PEARSON, V. (1982). Ultrastructural aspects of endomycorrhizas in the Ericaceae. III. Morphology of the dissociated symbionts and modifications occurring during their reassociation in axenic culture. *The New Phytologist*, **91**, 691-704.
- CALLOW, J. A., CAPACCIO, L. M., PARISH, G. & TINKER, P. B. (1978). Detection and estimation of polyphosphate in vesicular-arbuscular mycorrhizas. *The New Phytologist*, **80**, 125-134.
- CAPACCIO, L. C. M. & CALLOW, J. A. (1982). The enzymes of polyphosphate metabolism in vesicular-arbuscular mycorrhizas. *The New Phytologist*, **91**, 81-91.
- CHILVERS, G. A. & HARLEY, J. L. (1980). Visualization of phosphate accumulation in beech mycorrhizas. *The New Phytologist*, **84**, 319-326.
- COX, G., SANDERS, F. E., TINKER, P. B. & WILD, J. A. (1975). Ultrastructural evidence relating to host-endophyte transfer in vesicular-arbuscular mycorrhiza. In *Endomycorrhizas* (Ed. by F. E. Sanders, Barbara Mosse & P. B. Tinker), pp. 297-312. Academic Press, New York.
- COX, G., MORAN, K. J., SANDERS, F., NICKOLDS, C. & TINKER, P. B. (1980). Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. III. Polyphosphate granules and phosphorus translocation. *The New Phytologist*, **84**, 649-659.
- DUCLOS, J. L. & FORTIN, J. A. (1983). Effect of glucose and active charcoal on *in vitro* synthesis of ericoid mycorrhiza with *Vaccinium* spp. *The New Phytologist*, **94**, 95-102.
- DUDDRIDGE, J. & READ, D. J. (1982). An ultrastructural analysis of the development of mycorrhizas in *Rhododendron ponticum*. *Canadian Journal of Botany*, **60**, 2345-2356.
- GREENACRE, M. J. (1984). *Theory and Application of Correspondence Analysis*. Academic Press, New York.
- HARLEY, J. L. & MCCREADY, C. C. (1981). Phosphate accumulation in *Fagus* mycorrhizas. *The New Phytologist*, **84**, 75-80.
- HUGHES, D. E. & MUHAMMED, A. (1962). The metabolism of polyphosphate in bacteria. *Colloques Internationaux du Centre National de la Recherche Scientifique (Paris)*, **106**, 591-602.
- KEMPERS, A. J. (1975). Determination of submicro-quantities of plant available phosphate in aqueous soil extracts. *Plant and Soil*, **42**, 423-427.
- LING-LEE, M., CHILVERS, G. A. & ASHFORD, A. E. (1975). Polyphosphate granules in three different kinds of tree mycorrhiza. *The New Phytologist*, **75**, 551-554.
- MITCHELL, D. T. & READ, D. J. (1981). Utilization of inorganic and organic phosphates by the mycorrhizal endophytes of *Vaccinium macrocarpon* and *Rhododendron ponticum*. *Transactions of the British Mycological Society*, **76**(2), 255-260.
- PEARSON, V. & READ, D. J. (1973). The biology of mycorrhiza in the Ericaceae. I. The isolation of the endophyte and synthesis of mycorrhizas in aseptic culture. *The New Phytologist*, **72**, 371-379.
- PEARSON, V. & READ, D. J. (1975). The physiology of the mycorrhizal endophyte of *Calluna vulgaris*. *Transactions of the British Mycological Society*, **64**, 1-7.
- PETERSON, T. A., MUELLER, W. C. & ENGLANDER, L. (1980). Anatomy and ultrastructure of a *Rhododendron* root-fungus association. *Canadian Journal of Botany*, **58**, 2421-2433.
- READ, D. J. (1983). The biology of mycorrhiza in the Ericales. *Canadian Journal of Botany*, **61**, 985-1004.
- READ, D. J. & MITCHELL, D. T. (1983). Decomposition and mineralisation processes in mediterranean-type ecosystems and in heathlands of similar structure. In *Ecological Studies vol. 43. Mediterranean-Type Ecosystems: The Role of Nutrients* (Ed. by F. J. Kruger, D. T. Mitchell & J. U. M. Jarvis), pp. 208-232. Springer-Verlag, Berlin.



- REID, M. S. & BIELESKI, R. L. (1968). A simple apparatus for vertical flat-sheet polyacrylamide gel electrophoresis. *Analytical Biochemistry*, **22**, 374-381.
- ROBBINS, W. J. & WHITE, V. B. (1936). Limited growth and abnormalities in excised corn root tips. *Botanical Gazette*, **98**, 209-242.
- STRULLU, D. G., GOURRET, J. P., GARREC, J. P. & FOURCY, A. (1981). Ultrastructure and electron-probe microanalysis of the metachromatic vacuolar granules occurring in *Taxus mycorrhizas*. *The New Phytologist*, **87**, 537-545.
- STRULLU, D. G., HARLEY, J. L., GOURRET, J. P. & GARREC, J. P. (1982). Ultrastructure and microanalysis of the polyphosphate granules of the ectomycorrhizas of *Fagus sylvatica*. *The New Phytologist*, **92**, 417-423.
- STRULLU, D. G., HARLEY, J. L., GOURRET, J. P. & GARREC, J. P. (1983). A note on the relative phosphorus and calcium contents of metachromatic granules in *Fagus mycorrhizas*. *The New Phytologist*, **94**, 89-94.
- WHITE, J. A. & BROWN, M. F. (1979). Ultrastructure and X-ray analysis of phosphorus granules in a vesicular-arbuscular mycorrhizal fungus. *Canadian Journal of Botany*, **57**, 2812-2818.

July 18, 1973.

### Effect of Mycorrhizal Infection on Nitrogen and Phosphorus Nutrition of Ericaceous Plants

LARGE areas of the northern hemisphere are covered with nutrient-poor soils which support ericaceous plants. The roots of the most important representatives, *Calluna vulgaris* L. Hull. and species of *Vaccinium* and *Erica*, are always infected with an endotrophic mycorrhizal fungus. Much early work on these mycorrhizas was devoted to a controversy surrounding the nature of the infection and there is little known of the physiological role of the mycorrhizal association<sup>1</sup>.

Techniques, developed for the isolation of the endophyte and for the culture of mycorrhizal and non-mycorrhizal seedlings under aseptic conditions<sup>2</sup>, have been used to evaluate the influence of mycorrhizas on the nutrition of *C. vulgaris* and *Vaccinium macrocarpon* Ait. Small, uniform quantities of sterilized heathland soil were immersed in 0.5% water agar and aseptically germinated seedlings of the two species were transferred to the medium. Mycorrhizal seedlings were produced by inoculation of the soil with an isolate of the endophyte.

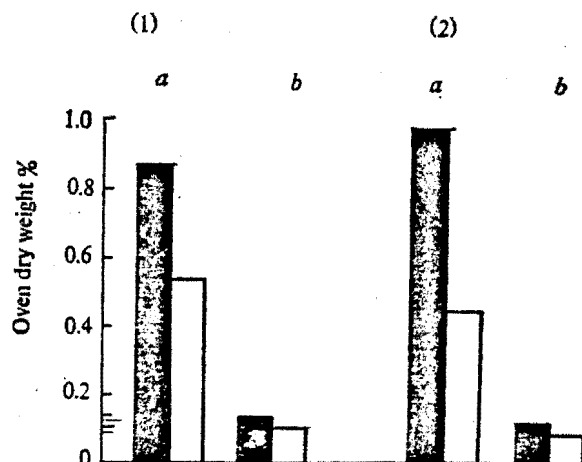


Fig. 1 The nitrogen, a, and phosphorus, b, contents of mycorrhizal (■) and non-mycorrhizal (□) seedlings of (1) *Calluna vulgaris* and (2) *Vaccinium macrocarpon* at the end of the first experiment.

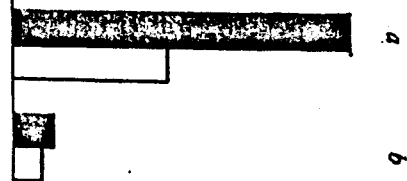
In the first experiment, plants of both species were collected six months after the mycorrhizal series was inoculated. Analyses of the nitrogen and phosphorus contents of shoots (Fig. 1) revealed a very significant ( $P < 0.001$ ) increase in the nitrogen content of mycorrhizal plants of both species and a less significant increase of phosphorus levels ( $P < 0.01$ ).

The nitrogen nutrition of *V. macrocarpon* was studied in greater detail in a second experiment in which root and shoot were analysed at the time of inoculation and after three and six month intervals (Table 1). The nitrogen content of mycorrhizal plants increased with time after inoculation until at the final collection it was nearly double that of sterile plants in both shoot and root. When expressed on a whole plant basis, the nitrogen content was again seen to be significantly increased by mycorrhizal infection and the final dry weight yield was significantly greater.

The enhanced nitrogen status of mycorrhizal plants may be a result of increased rates of mineralization and absorption of ammonium, the host benefiting from the transfer of amino acids synthesized by the endophyte. Such beneficial effects have been demonstrated in other mutualistic symbioses<sup>4</sup> and we are now studying these possibilities.

Whatever the cause of the greater nitrogen content of mycorrhizal plants, it will be of great importance to those species which are normally restricted to environments characterized by their low available nitrogen status.

(2)



torus, *b*, contents of mycorrhizal seedlings of (1) *Calluna vulgaris* at the end of the first year.

both species were collected and analysed. Analyses of contents of shoots (Fig. 1) show a significant increase in the nitrogen content in both species and a less significant increase in *Calluna*.

*macrocarpon* was studied in the year in which root and shoot contents were analysed and after three and six months after inoculation. The nitrogen content of mycorrhizal plants after inoculation until at least double that of sterile plants. This was significant on a whole plant basis, and the final dry weight yield was significantly increased.

mycorrhizal plants may be a result of the transfer of amino acids from the transfer of amino acids. Such beneficial effects of mutualistic symbioses and greater nitrogen content of plants of great importance to those related to environments characterized by low nitrogen status.

**Table 1** Nitrogen Content and Yield of Shoots and Roots of Mycorrhizal and Non-mycorrhizal\* Plants of *Vaccinium macrocarpon*

Growth stage	Shoot		Root		Total nitrogen N content mg/plant	Total yield Oven dry weight mg/plant	Appearance
	N content % oven dry weight	Yield mg oven dry weight	N content % oven dry weight	Yield mg oven dry weight			
1 Sterile seedlings at time of inoculation	0.74 ± 0.09	12.0 ± 2.3	0.61 ± 0.06	6.70 ± 0.06	0.11	18.70	
2 Three months after inoculation							
Mycorrhizal	0.80 ± 0.06	69.0 ± 9.0	0.68 ± 0.16	55.0 ± 4.0	0.88†	124.60	All leaves green. Plants vigorous. Apex yellow-green. Lower leaves reddening.
Non-mycorrhizal	0.51 ± 0.10	62.0 ± 3.2	0.51 ± 0.04	59.0 ± 7.0	0.62†	121.0	
3 Six months after inoculation							
Mycorrhizal	0.83 ± 0.04	171.0† ± 10.0	0.67 ± 0.12	64.0 ± 12.0	1.82†	235†	Leaves bright green. Stem red. Plants vigorous.
Non-mycorrhizal	0.46 ± 0.11	122.0† ± 7.0	0.40 ± 0.03	62.0 ± 6.1	0.81†	184†	Leaves purple, older ones senescent and browning. Stem brown woody.

\* Each figure represents a mean of fifteen plants except at stage one, where twelve plants were analysed.

† Indicate figures significantly different within the growth stage at  $P < 0.001$ .

We thank the NERC for support.

D. J. READ  
D. P. STRIBLEY

*Department of Botany,  
The University, Sheffield S10 2TN*

Received March 21, 1973.

- <sup>1</sup> Harley, J. L., *The Biology of Mycorrhiza* (Leonard Hill, London, 1969).
- <sup>2</sup> Pearson, V., and Read, D. J., *New Phytol.*, **72**, 371 (1973).
- <sup>3</sup> Melin, E., and Nilsson, H., *Svensk. Bot. Tidskr.*, **46**, 281 (1952).

# The FUNGAL COMMUNITY

---

ITS ORGANIZATION AND ROLE IN THE ECOSYSTEM

EDITED BY

*Donald T. Wicklow*

Northern Regional Research Center  
Agricultural Research  
Science and Education Administration  
U.S. Department of Agriculture  
Peoria, Illinois

*George C. Carroll*

Department of Biology  
University of Oregon  
Eugene, Oregon

1981

MARCEL DEKKER, INC.

New York and Basel

## Chapter 33

### ROLE OF ENDOMYCORRHIZAL FUNGI IN PHOSPHORUS CYCLING IN THE ECOSYSTEM

V. Gianinazzi-Pearson\* and S. Gianinazzi\*

Station de Physiopathologie Végétale  
Institut National de la Recherche Agronomique  
Dijon, France

#### I. INTRODUCTION

In natural ecosystems plants depend largely on the activity of soil microorganisms for the supply of mineral nutrients essential to their growth. It is evident that microorganisms that form symbiotic associations with plant roots, for example, nitrogen-fixing bacteria and mycorrhizal fungi, are particularly well placed to intervene in plant nutrition.

A distinguishing feature of mycorrhizal fungi is that after root infection part of the mycelium remains active in the soil. Plant roots provide them with an ecological niche with abundant substrate from which their hyphae extend outward through the soil and effectively explore a much greater volume than nonmycorrhizal roots. The two most important groups of mycorrhizal fungi consist of (1) those forming ectomycorrhizas which are characterized by mycelial sheaths around the roots and intercellular hyphal invasion of the root cortex and (2) those forming endomycorrhizas with a loose external hyphal network in the soil and extensive intracellular hyphal growth in the root cortex.

The beneficial effects of ectomycorrhizas on the growth and nutrition of tree species in soils of low nutrient status have been recognized for a long time (Mitchell et al., 1937; McComb, 1938), and the role of ectomycorrhizal fungi in supplying mineral nutrients to the host plant has been conclusively established (Melin and Nilsson, 1950, 1952, 1953a,b; Melin et al., 1958; Harley, 1969). It is only within the last few years, however, that the potential importance of endomycorrhizas in the growth and mineral nutrition of their host plants has been widely appreciated (Mosse, 1973; Gianinazzi-Pearson, 1976). Two groups of endomycorrhizas<sup>†</sup> are able to benefit their host plants in improving mineral nutrient uptake: the ericoid and the vesicular-arbuscular mycorrhizas. This chapter deals in particular with the role of the fungi that form these endomycorrhizas in phosphorus uptake by plants and discusses the importance of this role in the cycling of phosphorus in the ecosystem.

---

\* Station d'Amélioration des Plantes, Institut National de la Recherche Agronomique, Dijon, France.

<sup>†</sup> A third group, found in the Orchidaceae and concerned with the carbohydrate nutrition of developing seedlings, is not considered here.

## II. ENDOMYCORRHIZAL INFECTIONS: ECOLOGY, MORPHOLOGY, AND ENDOPHYTE TAXONOMY

Ericoid mycorrhizas are restricted to genera of the Ericaceae, of which *Calluna*, *Vaccinium*, and *Erica* are examples. These plants occur widely as dominant and codominant members of calcifuge plant communities and are normally associated with mor-humus soils of low nutrient status. The endomycorrhizal fungi can be isolated and cultured axenically (Pearson and Read, 1973a), and one true mycorrhizal isolate has been identified as *Pezizella erica* sp. nov. (Read, 1974). It is probable that all ericoid endophytes will ultimately be recognized as Ascomycetes of this or a related genus. Endomycorrhizas are formed annually with development of the lateral hair roots of the host plant. Endophytic hyphae present in the soil or originating from previously infected roots penetrate the host cells after formation of appressoria and by repeated branching develop compact intracellular mycelial complexes, or "hyphal coils," in the outer cortical cells (Fig. 1a). These intracellular hyphae appear to be separated from the host cytoplasm by the surrounding intact host plasmalemma (Fig. 1b). Nutrient exchange is thought to take place by lysis of these intracellular hyphae, but this has not yet been demonstrated. In young seedlings the extent of mycorrhizal infection can reach up to 70% of the total root system and the number of hyphal entry points well over 1000 per centimeter of root (Read and Stribley, 1975). The mycelium spreads around the root and into the soil, establishing frequent hyphal connections between infected host cells and soil particles around the root.

Vesicular-arbuscular (VA) mycorrhizas, unlike ericoid mycorrhizas, are not limited to any one plant family and have an exceptionally wide range of hosts and habitats. There are only a few families where they are not found, and these include some forming ectomycorrhizas or non-VA endomycorrhizas and those not forming mycorrhizas at all (Chenopodiaceae and Cruciferae). VA mycorrhizas are formed by phycomycetous fungi which cannot be cultured axenically but which, on the basis of their spore morphology, have been identified as members of the genera *Glomus*, *Gigaspora*, *Acaulospora*, and *Sclerocystis* of the Endogonaceae (Gerdemann and Trappe, 1974). There is a marked lack of host specificity among the different VA endophyte strains or species. Typical aspects of VA infections are shown in Figs. 1c, d, and e for soybean, onion, and clover roots, respectively. An infecting hypha, originating from a spore or previously infected root in the soil, enters the root without forming a well-defined appressorium, then ramifies rapidly and spreads intercellularly along the inner layers of the cortex (Fig. 1c). At intervals hyphae penetrate the cortical cells and form the highly branched haustoria-like structures known as arbuscules (Fig. 1d). These intracellular hyphae do not penetrate into the host cytoplasm but remain enveloped by the host plasmalemma (see Fig. 6a in Sec. IV), thus creating a large surface area of contact between the fungus and the host cell (Cox and Sanders, 1974; Cox and Tinker, 1976; Dexheimer et al., 1979). Although there is no direct proof, this is generally regarded as the site of transfer of material between the symbionts. Lipid-containing vesicles, which are probably storage organs, may form as the infection ages (Fig. 1e). The extent of VA infection varies considerably according to the host plant, the endo-



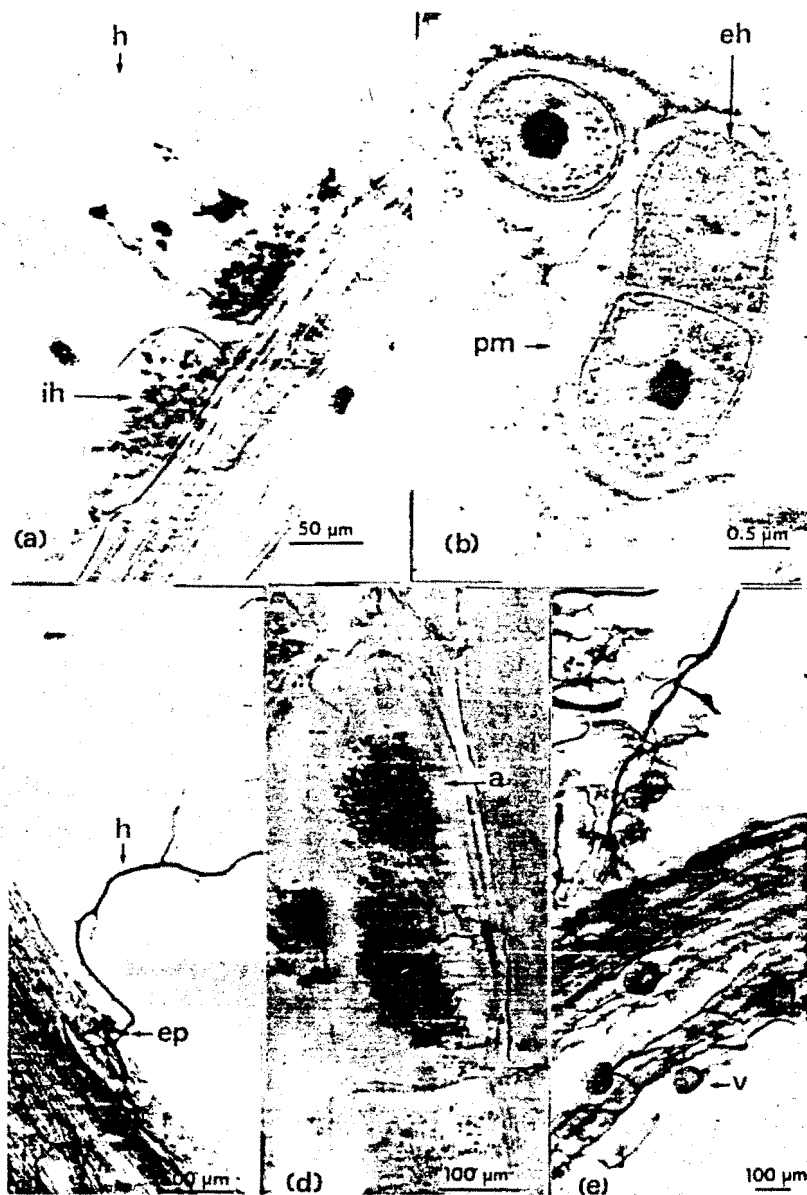


Fig. 1 Ericoid (a,b) and VA (c,d,e) endomycorrhizal infections. (a) Field infected hair root of *Calluna vulgaris* showing penetrating hyphae (h) and intracellular hyphal complexes (ih). (b) Electron micrograph of endophytic hyphae (eh) surrounded by host plasmalemma (pm) in a cortical root cell of *C. vulgaris* (by courtesy of P. Bonfante-Fasolo, CSMT-CNR, Turin). (c) Soyabean root infected with *Glomus mosseae* showing external hypha (h), entry point (ep) and intercellular mycelium. (d) Arbuscule (a) of *G. mosseae* in onion root. (e) Vesicles (v) and intercellular hyphae of *G. mosseae* in clover root. External vesicles and mycelium are also present.

phyte strain, and the habitat, but it may attain 95% of the root system (Khan, 1975).

Hyphae spreading along the root surface make new entry points [2-20 per cm (Mosse,

Table 1 Responses to ericoid and vesicular-arbuscular (VA) endomycorrhizas

Plant and duration of experiment	Medium	Type of endomycorrhiza	Mycorrhizal status <sup>a</sup>	Yield (mg dry weight per plant)	Reference
<i>Calluna vulgaris</i> 12 weeks	Sterile sand	Ericoid	M	2.5	Pearson (1971)
			NM	0.6	
<i>Vaccinium macrocarpon</i> 6 months	Sterile soil/agar	Ericoid	M	235	Read and Stribley (1973)
			NM	184	
<i>Lycopersicon esculentum</i> (30) 9 weeks	Sterile sand	VA	M	535	Daft and Nicolson (1966)
			NM	198	
<i>Coprosma robusta</i> 3 months	Irradiated soil	VA	M	160 <sup>b</sup>	Hayman and Mosse (1971)
			NM	20	
<i>Allium cepa</i> 10 weeks	Irradiated soil	VA	M	255 <sup>b</sup>	Gianinazzi-Pearson and Gianinazzi (1978)
			NM	52	

<sup>a</sup>M, mycorrhizal; NM, nonmycorrhizal.

<sup>b</sup>Shoots only.

Stribley and Read, 1974), and it is thought that the mycorrhizas aid in the uptake of both these elements by the host plants. In VA infections, however, the only consistently important differences between mycorrhizal and nonmycorrhizal plants is the higher phosphorus content of the former (Gerdemann, 1964; Holevas, 1966; Bowen and Theodorou, 1967; Gray and Gerdemann, 1967; Sanders and Tinker, 1971; Sanders et al., 1977). Although other elements such as nitrogen (Ross, 1971), zinc (Gilmore, 1971), and sulfur (Gray and Gerdemann, 1973) have been shown to be involved occasionally, phosphorus is regarded as by far the most important nutrient concerned in the growth responses.

Studies of phosphate uptake from isotopically labeled solutions have shown that this higher accumulation of phosphorus in endomycorrhizal plants is the result of an enhanced uptake by infected roots (Table 2). Roots of *Calluna* seedlings exposed to labeled phosphate solution have 3-4.5 times more activity when mycorrhizal; VA mycorrhizal clover and *Liriodendron* roots have about twice as much activity as nonmycorrhizal roots. The rate of transfer of this absorbed phosphorus to the shoot differs in the two types of endomycorrhiza. In mycorrhizal ericaceous plants there is some accumulation of phosphorus-32 in the roots with a relatively slow release to the shoots (Pearson, 1971; Pearson and Read, 1973b), whereas in VA-infected plants there is a rapid translocation of the element to the aerial portions of the plant (Gray and Gerdemann, 1969; Rhodes and Gerdemann, 1975). These results demonstrate clearly that

Table 3 Effect of ericoid and VA mycorrhizas on acid phosphatase activity of detached roots

Plant	Type of mycorrhiza	Mycorrhizal status <sup>a</sup>	Surface acid phosphatase activity ( $\mu\text{mol p-nitrophenol mg}^{-1}$ dry weight of root)
<i>Calluna vulgaris</i>	Ericoid	M	14.6 <sup>b</sup>
		NM	8.0 <sup>b</sup>
<i>Allium cepa</i>	VA	M	1.5
		NM	1.6

<sup>a</sup>M, mycorrhizal; NM, nonmycorrhizal

<sup>b</sup>Significantly different at 5% level.

and Mosse, 1972; Mosse et al., 1973). It has therefore been concluded that both VA mycorrhizal and nonmycorrhizal plants draw their phosphate from the same source and, from measurements of the specific activity of the soil solutions (J. C. Fardeau, personal communication, 1978), that this source is the soil solution or adsorbed phosphate in equilibrium with it. The fact that VA mycorrhiza formation does not modify root surface acid phosphatase activity (Table 3), which is believed to contribute to the mobilization of insoluble organophosphorus compounds by plants (Weissflog and Mengdehl, 1933; Rogers et al., 1940; Saxena, 1964; Wild and Oke, 1966), provides further evidence to support this conclusion. The VA mycorrhizal effect thus appears to be due to a more efficient absorption of available phosphorus and not solubilization. The growth responses of mycorrhizal plants in the presence of relatively insoluble inorganic phosphates could be due to a more efficient uptake of the chemically dissociated ions drawn into solution from solid phase phosphate as the solution phosphate is depleted. This would explain the decline in the relative advantage of VA mycorrhizal over nonmycorrhizal plants in the presence of large amounts of bonemeal (Daft and Nicolson, 1966, 1972).

There is evidence that ericoid mycorrhizas, on the contrary, are active in the mobilization of insoluble organophosphorus compounds in the soil; mycorrhizal roots have a much higher surface acid phosphatase activity than do nonmycorrhizal roots (Table 3). It seems possible therefore that mycorrhizal infection may enhance phosphorus nutrition in ericaceous plants by both a more efficient uptake of available phosphorus and an increased utilization of insoluble phosphorus complexes in the soil. Further work is clearly necessary to verify this point.

#### IV. ROLE OF ENDOMYCORRHIZAL FUNGI

Several hypotheses have been postulated to explain the increased phosphorus uptake of plants following endomycorrhizal infection (Harley, 1969; Sanders and Tinker,

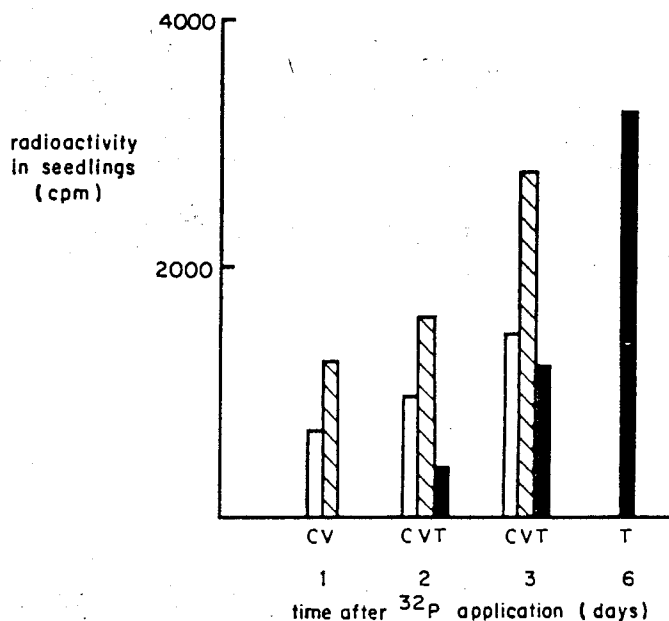


Fig. 4 Phosphorus-32 translocation to seedlings by ericoid (C, V) and VA (T) mycorrhizal fungi. Key: C, *Calluna vulgaris* (cpm/whole seedling  $\times 10^2$ ) (Pearson and Read, 1973b); V, *Vaccinium oxycoccos* (cpm/mg fresh weight of shoot  $\times 10$ ) (Pearson and Read, 1973b); T, *Trifolium repens* (cpm/shoot) (V. Pearson and P. B. H. Tinker, unpublished data).

been reported for ericoid fungi (Pearson and Read, 1973b) and up to 8 cm, in a different system, for VA fungi (Rhodes and Gerdemann, 1975). The ramifying external mycelia of endomycorrhizal fungi in the soil can thus provide the host plant with a means of absorbing available phosphorus from nondepleted sources in the soil at an appreciable distance from the root.

Using the aforementioned system, Pearson and Tinker (1975) measured fluxes of  $0.3$  to  $1.0 \times 10^{-9}$  mol P  $\text{cm}^{-2} \text{sec}^{-1}$  in hyphae of a VA endophyte at some distance from the host root, values which are not very different from that of  $3.8 \times 10^{-8}$  mol P  $\text{cm}^{-2} \text{sec}^{-1}$  computed theoretically by Sanders and Tinker (1973) for the same fungus. Since these values are too high to be explained by simple diffusion, an active transport mechanism must be involved and, with the finding of polyphosphate granules in the vacuoles of VA fungi (Cox et al., 1975), Tinker (1975, p. 339) has proposed "cytosis, plus bulk flow, with loading and unloading of polyphosphate into vacuoles as the method of varying the phosphorus concentration of the streaming protoplasm." There is now evidence that the subsequent transfer of phosphorus from the fungus into the host cell is also an active process, taking place across the living interface (Cox and Tinker, 1976), and that it does not result from digestion of the fungus as previously believed.

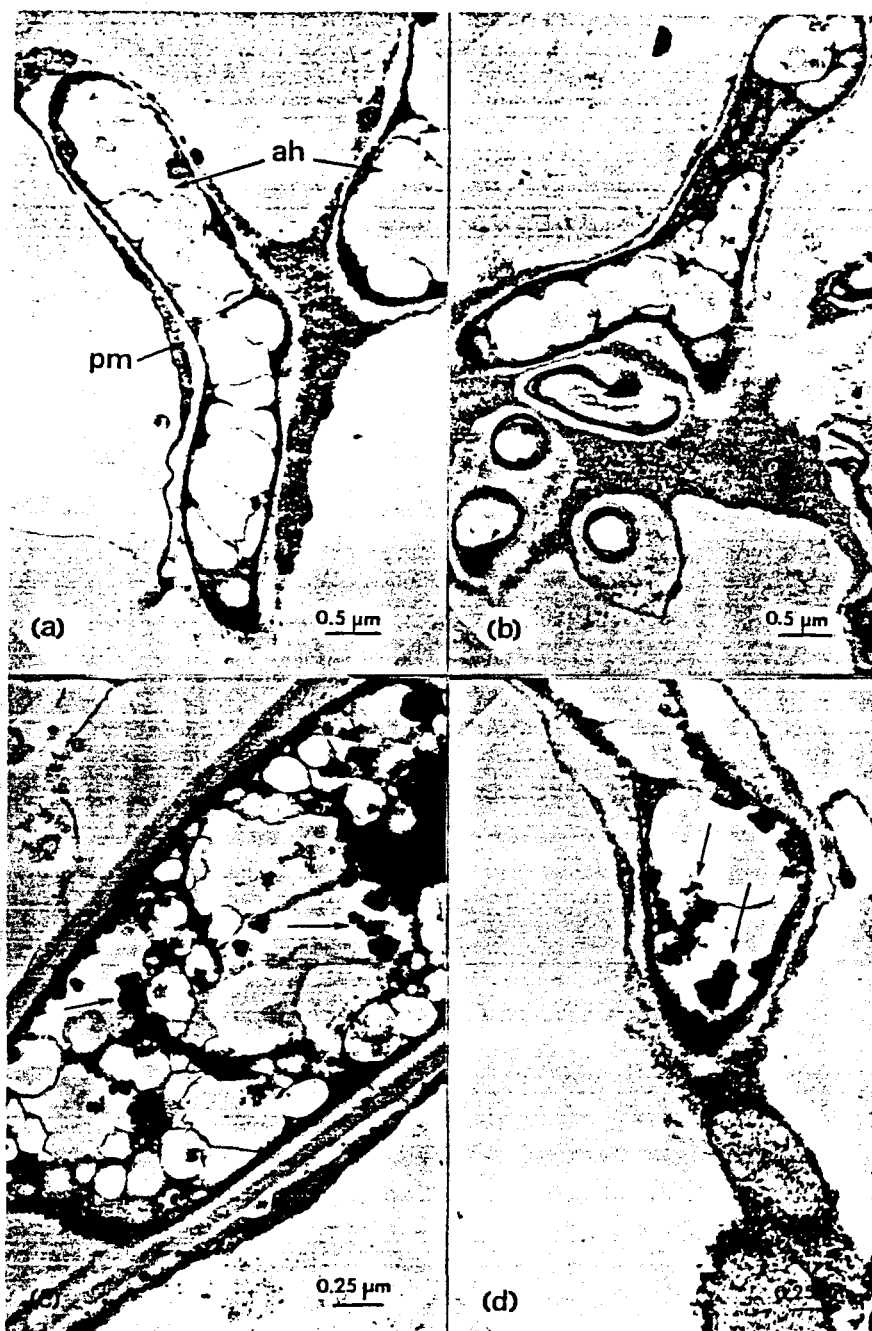


Fig. 6 Electron micrographs of localization of alkaline phosphatase activity within hyphae of the VA mycorrhizal fungus *G. mosseae* in onion roots. Black precipitate (arrows) indicates enzyme activity within fungal vacuoles. (a) Substrate omitted (ah, arbuscular hyphae; pm, host plasmalemma). (b) Substrate ( $\alpha$ -naphthyl phosphate) plus KCN (alkaline phosphatase inhibitor). (c)  $\alpha$ -Naphthyl phosphatase activity. (d)  $\beta$ -Glycerophosphatase activity.

## REFERENCES

- Asimi, S., Gianinazzi-Pearson, V., Gianinazzi, S., Obaton, M., and Berthau, S. (1978). Interactions entre les endomycorhizes à vésicules et arbuscules (VA) et le *Rhizobium* chez la soja. 103<sup>e</sup> Congr. Nat. Soc. Sav., Vol. 1, pp. 247-256.
- Bannister, P., and Norton, W. M. (1974). The response of mycorrhizal rooted cuttings of heather (*Calluna vulgaris* (L) Hull) to variations in nutrient and water regimes. *New Phytol.* 73: 81-88.
- Bertheau, Y. (1977). Etudes des phosphatases solubles des endomycorhizes à vésicules et arbuscules. D.E.A. thesis, University of Dijon, France.
- Beslow, D. T., Hacskeylo, E., and Melhuish, J. H. (1970). Effects of environment on beaded root development in red maple. *Bull. Torrey Bot. Club* 97: 248-252.
- Bowen, G. D., and Theodorou, C. (1967). Studies on phosphate uptake by mycorrhizas. *Proc. 14th IUFRO Congr.* 24: 116-138.
- Brook, P. J. (1952). Mycorrhiza of *Pernettya macrostigma*. *New Phytol.* 51: 388-397.
- Burgeff, H. (1961). *Mikrobiologie des Hochmoores*. Fischer, Stuttgart.
- Cosgrove, D. J., and Tate, M. E. (1963). The occurrence of neo-inositol hexaphosphate in soil. *Nature* 200: 568-569.
- Cox, G., and Sanders, F. (1974). Ultrastructure of the host-fungus interface in a vesicular-arbuscular mycorrhiza. *New Phytol.* 73: 901-912.
- Cox, G., and Tinker, P. B. (1976). Translocation and transfer of nutrients in vesicular-arbuscular mycorrhiza. I. The arbuscule and phosphorus transfer: A quantitative ultrastructural study. *New Phytol.* 77: 371-378.
- Cox, G. C., Sanders, F. E. T., Tinker, P. B., and Wild, J. (1975). Ultrastructural evidence relating to host-endophyte transfer in a vesicular-arbuscular mycorrhiza. In *Endomycorrhizas*, F. E. Sanders, B. Mosse, and P. B. Tinker (Eds.). Academic Press, New York, pp. 297-312.
- Daft, M. J., and Nicolson, T. H. (1966). Effect of *Endogone* mycorrhiza on plant growth. *New Phytol.* 65: 343-350.
- Daft, M. J., and Nicolson, T. H. (1972). Effect of *Endogone* mycorrhiza on plant growth. IV. Quantitative relationships between the growth of the host and the development of the endophyte in tomato and maize. *New Phytol.* 71: 287-295.
- Dexheimer, J., Gianinazzi, S., and Gianinazzi-Pearson, V. (1979). Ultrastructural cytochemistry of the host-fungus interfaces in the endomycorrhizal association *Glomus mosseae*/*Allium cepa*. *Z. Pflanzenphysiol.* 92: 191-206.
- Fitter, A. H. (1977). Influence of mycorrhizal infection on competition for phosphorus and potassium by two grasses. *New Phytol.* 79: 119-125.
- Freisleben, R. (1936). Weitere Untersuchungen über die Mykotrophie der Ericaceen. *Jahrb. Wiss. Bot.* 82: 413-459.
- Gerdemann, J. W. (1964). The effect of mycorrhiza on the growth of maize. *Mycologia* 56: 342-349.
- Gerdemann, J. W., and Trappe, J. M. (1974). The Endogonaceae of the Pacific Northwest. *Mycol. Memoir* 5: 75 pp.
- Gianinazzi, S., Gianinazzi-Pearson, V., and Dexheimer, J. (1979). Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza. III. Ultrastructural localisation of acid and alkaline phosphatase in onion roots infected by *Glomus mosseae* Nichol. & Gerd. *New Phytol.* 82: 127-132.
- Gianinazzi-Pearson, V. (1976). Les mycorhizes endotrophes: état actuel des connaissances et possibilités d'application dans la pratique culturale. *Ann. Phytopath.* 8: 249-256.

- Mitchell, H. L., Finn, R. F., and Rosendhal, R. O. (1937). The relation between mycorrhizae and the growth and nutrient absorption of coniferous seedlings in nursery beds. *Black Rock For. Papers* 1: 58-73.
- Morrison, T. M. (1957). Host-endophyte relationship in mycorrhiza of *Pernettya macrostigma*. *New Phytol.* 56: 247-257.
- Mosse, B. (1959). The regular germination of resting spores and some observations on the growth requirements of *Endogone* species causing vesicular-arbuscular mycorrhizas. *Trans. Brit. Mycol. Soc.* 42: 274-286.
- Mosse, B. (1973). Advances in the study of vesicular-arbuscular mycorrhiza. *Ann. Rev. Phytopath.* 11: 171-196.
- Mosse, B., and Bowen, G. D., quoted by Bowen and Theodorou (1967), above.
- Mosse, B., Hayman, D. S., and Arnold, D. (1973). Plant growth responses to vesicular-arbuscular mycorrhiza. V. Phosphate uptake by three plant species from P-deficient soils labelled with  $^{32}\text{P}$ . *New Phytol.* 72: 809-815.
- Murdoch, C. L., Jackobs, J. A., and Gerdemann, J. W. (1967). Utilization of phosphorus sources of different availability by mycorrhizal and non-mycorrhizal maize. *Plant Soil* 27: 329-334.
- Nieuwdorp, P. J. (1969). Some investigations on the mycorrhiza of *Calluna*, *Erica*, and *Vaccinium*. *Acta Bot. Neerl.* 18: 180-196.
- North, J., and Lewis, D. (1971). Phosphatases of *Coprinus lagopus*: The conditions for their production and the genetics of the alkaline phosphatase. *Genet. Res.* (Cambridge) 18: 153-166.
- Nye, P. H. (1969). The soil model and its application to plant nutrition. In *Ecological Aspects of the Mineral Nutrition of Plants*, *Symp. Brit. Ecol. Soc.* 9: 201-213.
- Pearson, V. (1971). The biology of mycorrhiza in the Ericaceae. Ph.D. thesis, University of Sheffield, England.
- Pearson, V., and Read, D. J. (1973a). The biology of mycorrhiza in the Ericaceae. I. The isolation of the endophyte and synthesis of mycorrhiza in aseptic culture. *New Phytol.* 72: 371-379.
- Pearson, V., and Read, D. J. (1973b). The biology of mycorrhiza in Ericaceae. II. The transport of carbon and phosphorus by the endophyte and the mycorrhiza. *New Phytol.* 72: 1325-1331.
- Pearson, V., and Read, D. J. (1975). The physiology of the mycorrhizal endophyte of *Calluna vulgaris*. *Trans. Brit. Mycol. Soc.* 64: 1-7.
- Pearson, V., and Tinker, P. B. H. (1975). Measurement of phosphorus fluxes in the external hyphae of endomycorrhizas. In *Endomycorrhizas*, F. E. Sanders, B. Mosse, and P. B. Tinker (Eds.). Academic Press, New York, pp. 277-287.
- Read, D. J. (1974). *Pezizella ericae* sp. nov. the perfect state of a typical mycorrhizal endophyte of Ericaceae. *Trans. Brit. Mycol. Soc.* 63: 381-383.
- Read, D. J., and Stribley, D. P. (1973). Effect of mycorrhizal infection on nitrogen and phosphorus nutrition of ericaceous plants. *Nature* 244: 81-82.
- Read, D. J., and Stribley, D. P. (1975). Some mycological aspects of the biology of mycorrhiza in the Ericaceae. In *Endomycorrhizas*, F. E. Sanders, B. Mosse, and P. B. Tinker (Eds.). Academic Press, New York, pp. 105-117.
- Rhodes, L. H., and Gerdemann, J. W. (1975). Phosphate uptake zones of mycorrhizal and non-mycorrhizal onions. *New Phytol.* 75: 555-561.
- Rogers, H. T., Pearson, R. W., and Pierre, N. H. (1940). Absorption of organophosphates by corn and tomato plants and the mineralizing action of exoenzyme systems of growing roots. *Soil. Sci. Soc. Amer. Proc.* 5: 285-291.
- Ross, J. P. (1971). Effect of phosphate fertilization on yield of mycorrhizal and non-mycorrhizal soybeans. *Phytopathol.* 61: 1400-1403.

- Sanders, F. E., and Tinker, P. B. (1971). Mechanism of absorption of phosphate by *Endogone mycorrhizas*. *Nature* 233: 278-279.
- Sanders, F. E. T., and Tinker, P. B. H. (1973). Phosphate flow into mycorrhizal roots. *Pestic. Sci.* 4: 385-395.
- Sanders, F. E. T., Tinker, P. B., Black, R. L. B., and Palmerley, S. M. (1977). The development of endomycorrhizal root systems. I. Spread of infection and growth-promoting effects with four species of vesicular-arbuscular endophyte. *New Phytol.* 78: 257-268.
- Saxena, S. N. (1964). Phytase activity of plant roots. *J. Exp. Bot.* 15: 654-658.
- Stribley, D. P., and Read, D. J. (1974). The biology of mycorrhiza in the Ericaceae. IV. The effect of mycorrhizal infection on uptake of  $^{15}\text{N}$  from labelled soil by *Vaccinium macrocarpon* Ait. *New Phytol.* 73: 1149-1155.
- Stribley, D. P., and Read, D. J. (1976). The biology of mycorrhiza in the Ericaceae. VI. The effects of mycorrhizal infection and concentration of ammonium nitrogen on growth of cranberry (*Vaccinium macrocarpon* Ait.) in sand culture. *New Phytol.* 77: 63-72.
- Stribley, D. P., Read, D. J., and Hunt, R. (1975). The biology of mycorrhiza in the Ericaceae. V. The effects of mycorrhizal infection, soil type and partial soil sterilization (by gamma-irradiation) on growth of cranberry (*Vaccinium macrocarpon* Ait.). *New Phytol.* 75: 119-130.
- Tinker, P. B. H. (1975). Effects of vesicular-arbuscular mycorrhizas on higher plants. In *Symbiosis, Symp. Soc. Exp. Biol.* 29: 325-349.
- Weissflog, J., and Mengdehl, H. (1933). Studien zum Phosphorstoffwechsel in den höheren Pflanzen. III. Aufnahme und Verwertbarkeit organischer Phosphorsäureverbindungen durch die Pflanze. *Planta* 19: 182-241.
- Wild, A., and Oke, O. L. (1966). Organic phosphate compounds in calcium chloride extracts of soil: Identification and availability to plants. *J. Soil Sci.* 17: 356-371.

## REFERENCES ADDED IN PROOF

- Asimi, S., Gianinazzi-Pearson, V., and Gianinazzi, S. (1980). Influence of increasing soil phosphorus levels on interactions between vesicular-arbuscular mycorrhizae and *Rhizobium* in soybeans. *Can. J. Bot.* 58: 2200-2206.
- Bonfante-Fasolo, P., and Gianinazzi-Pearson, V. (1979). Ultrastructural aspects of endomycorrhiza in the Ericaceae. I. Naturally infected hair roots of *Calluna vulgaris* L. Hull. *New Phytol.* 83: 739-744.
- Callow, J. A., Capaccio, L. C. M., Parish, G., and Tinker, P. B. (1978). Detection and estimation of polyphosphate in vesicular-arbuscular mycorrhizas. *New Phytol.* 80: 125-134.
- Cooper, K. M., and Tinker, P. B. (1978). Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. II. Uptake and translocation of phosphorus, zinc and sulphur. *New Phytol.* 81: 43-52.
- Cox, G., Moran, K. J., Sanders, F., Nockolds, C., and Tinker, P. B. (1980). Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. III. Polyphosphate granules and phosphorus translocation. *New Phytol.* 84: 649-659.
- Cress, W. A., Throneberry, G. O., and Lindsey, D. L. (1979). Kinetics of phosphorus absorption in mycorrhizal and nonmycorrhizal tomato roots. *Plant Physiol.* 64: 484-487.